Hypoxia and the regulation of mitogen-activated protein kinases: gene transcription and the assessment of potential pharmacologic therapeutic interventions

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Abstract

Oxygen is an environmental/developmental signal that regulates cellular energetics, growth, and differentiation processes. Despite its central role in nearly all higher life processes, the molecular mechanisms for sensing oxygen levels and the pathways involved in transducing this information are still being elucidated. Altering gene expression is the most fundamental and effective way for a cell to respond to extracellular signals and/or changes in its microenvironment. During development, the expression of specific sets of genes is regulated spatially (by position/morphogenetic gradients) and temporally, presumably via the sensing of molecular oxygen available within the microenvironment. Regulation of signaling responses is governed by transcription factors that bind to control regions (consensus sequences) of target genes and alter their expression in response to specific signals. Complex signal transduction during hypoxia (deficiency of oxygen in inspired gases or in arterial blood and/or in tissues) involves the coupling of ligand–receptor interactions to many intracellular events. These events basically include phosphorylations by tyrosine kinases and/or serine/threonine kinases, such as those of mitogen-activated protein kinases (MAPKs), a superfamily of kinases responsive to stress nonhomeostatic conditions. Protein phosphorylations imposed during hypoxia change enzyme activities and protein conformations, and the eventual outcome is rather complex, comprising of an

**Abbreviations:** NAC, N-acetyl-L-cysteine; AP, activating protein; ATF, activating transcription factor; bFGF, basic fibroblast growth factor; CaM, calmodulin; PKM, Ca²⁺/CaM-dependent protein kinase; CT, cardiomyophrin; COXII, cytochrome oxidase I; DTT, dithiothreitol; dHIF, *Drosophila* HIF; Draf, *Drosophila* Raf; EGF, epidermal growth factor; ERT kinase, EGF receptor threonine kinase; EPO, erythropoietin; ECM, extracellular matrix; ERK, extracellular signal-regulated kinase; GPCR, G-protein-coupled receptor; GSH, glutathione; hep, hemipterous; HGF, hepatocyte growth factor; HHV, human herpes virus; HMEC, human microvascular endothelial cells; HIF, hypoxia-inducible factor; HPTF, HIF proteasome-targeting factor; HRE, hypoxia response element; IκB, inhibitory κB; IFN, interferon; IL, interleukin; JNK, Jun N-terminal kinase; KSHV, Kaposi’s sarcoma-associated herpes virus; LPS, lipopolysaccharide endotoxin; NMDA, N-methyl-D-aspartate; MAP-2 kinase, microtubule-associated protein-2 kinase; MAPK, mitogen-activated protein kinase; MKP, MAPK phosphatase; MEKK, MKK kinase; MAPKAP-K, mitogen-activated protein kinase-activated protein kinase; MBP kinase, myelin basic protein kinase; NGF, nerve growth factor; NO, nitric oxide; NF-κB, nuclear factor-κB; NIK, NF-κB-inducing kinase; NLS, nuclear localization signal; OA, okadaic acid; Ppase-1, phosphatase-1; PDGF, platelet-derived growth factor; PC, preconditioning; PKA, protein kinase A; PKC, protein kinase C; PTK, protein tyrosine kinase; puc, puckered; RTK, receptor tyrosine kinase; RHD, Rel homology domain; rho, rhomboid; RSK, ribosomal S6 protein kinase; SRF, serum response factor; spi, Spitz; SAPK, stress-activated protein kinase; Sp, substance P; SOD, superoxide dismutase; Tor, torso; TGF, transforming growth factor; TNF, tumor necrosis factor; TyrK, tyrosine kinase; UB, ubiquitin; VEGF, vascular endothelial growth factor; vn, vein; pVHL, von Hippel–Lindau protein.

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alteration in cellular activity and changes in the programming of genes expressed within the responding cells. These molecular changes serve as signals that are crucial for cell survival under contingent conditions imposed during hypoxia. This review correlates current concepts of hypoxic sensing pathways with hypoxia-related phosphorylation mechanisms mediated by MAPKs via the genetic and pharmacologic regulation/manipulation of specific transcription factors and related cofactors.

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**Keywords:** Cell signaling; Development; Disease; Gene regulation; Hypoxia; Kinase; MAPK; Transcription factors

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### 1. Introduction

Gene regulation (activation/repression) is a complex biological process that results from molecular interactions among nuclear protein factors (transcription factors) and DNA consensus control sequences [1–5]. These protein–DNA interactions often occur as a result of an extracellular stimulus that is transmitted to the nucleus by a specific signal transduction pathway(s). Although numerous stimuli that regulate gene expression have been identified, perhaps none is more intriguing than reduced oxygen (hypoxia) [2,6–10]. Hypoxia-induced gene expression has been implicated in a number of physiological processes, including erythropoiesis, carotid body chemoreceptor function, and angiogenesis, all of which enhance the delivery of oxygen to tissues [1,9,10].

Oxygen is a unique stimulus that readily diffuses throughout the cell; thus, hypoxia may regulate gene expression by a variety of different mechanisms in different cell types. Major challenges, however, include: (i) identification of oxygen sensory mechanisms; (ii) further identification and characterization of oxygen-regulated signal transduction pathways; (iii) identification of additional genes that are regulated by hypoxia; and (iv) understanding the role that these genes play in regulating the response to hypoxia. Such information will provide new insights into hypoxia-regulated physiological (signaling and developmental) and pathological (disease) processes [1–4,6–10]. In this review, I particularly stress emphasis on the current understanding of the role of hypoxia in regulating the mitogen-activated protein kinase (MAPK) pathways via upstream and downstream mechanisms.
2. MAPK signaling modules and pathways: a network recap and current concepts

2.1. MAPK signaling pathways as viewed through their identification and bifurcations

Signal transduction at the cellular level refers to the movement of signals from outside (extracellular) the cell to inside (intracellular) the cell [11–18]. The movement of signals can be simple, like that associated with receptor molecules of the acetylcholine class receptors that constitute channels, which, upon ligand interaction, allow signals to be passed in the form of small ion movement, either into or out of the cell [11–13,18]. These ion movements result in changes in the electrical potential of the cells that, in turn, propagates the signal spatially along the cell. More complex signal transduction, furthermore, involves the coupling of ligand–receptor interactions to many intracellular events. These events include phosphorylations by tyrosine kinases (TyrKs) and/or serine/threonine kinases [11–15,17,18]. Protein phosphorylation changes enzyme activities and protein conformations. The eventual outcome is an alteration in cellular activity and changes in the programming of genes expressed within the responding cells. Phosphorylation and dephosphorylation mechanisms in the regulation of transcription are depicted in Fig. 1.

MAPKs were identified by virtue of their activation in response to growth factor stimulation of cells in culture, hence the name MAPKs (Fig. 2) [19–25]. MAPKs have similar biochemical properties, immuno-cross-reactivities, amino acid sequence, and ability to in vitro phosphorylate similar substrates. Maximal MAPK activity requires that both tyrosine and threonine residues are phosphorylated. This indicates that MAPKs act as switch kinases that transmit information of increased intracellular tyrosine phosphorylation to that of serine/threonine phosphorylation [26–28].

Although MAPK activation was first observed in response to the activation of epidermal growth factor (EGF), platelet-derived growth factor (PDGF), nerve growth factor (NGF), and insulin and insulin-like receptors, other cellular stimuli such as T-cell activation (which signals through the Lck TyrK); phorbol esters (which function through activation of protein kinase C, or PKC); thrombin, bombesin, and brady-

![Fig. 1. A model of gene regulation where the switch on/off mediates a series of phosphorylation/dephosphorylation steps regulated by kinases and phosphatases, respectively. This sequential propagation of signals occurs in response to a stimulus over a prespecified period of time.](image-url)
kinin (which function through G-proteins); as well as N-methyl-D-aspartate (NMDA) receptor activation and electrical stimulation rapidly induce tyrosine phosphorylation of MAPKs [20,22–24,26–28]. MAPKs are, however, not the direct substrates for receptor tyrosine kinases (RTKs) or receptor-associated TyrKs, but are in fact activated by an additional class of kinases termed MAPK kinases (MKKs) and MAPK kinase kinases (MAPKK kinases). One of the MAPKs has been identified as the proto-oncogenic serine/threonine kinase, Raf [29–42]. Ultimate targets of the MAPKs are several transcriptional regulators such as serum response factor (SRF) and the proto-oncogenes Fos, Myc, and Jun, as well as members of

![Fig. 2. The modules and various components of the MAPK signaling pathways. The cellular response to growth factors, inflammatory cytokines, and other mitogens is often mediated by receptors that either are G-protein-linked or are intrinsic protein tyrosine kinases. The binding of the ligand to receptor tyrosine kinases induces dimerization and autophosphorylation (activation) of the kinase. The activated tyrosine kinase binds to, and phosphorylates, an adaptor protein, such as Grb2, which, in turn, activates a guanine nucleotide exchange factor, such as mSOS, which, in turn, activates a small GTP-binding protein, such as Ras or Rac. The GTP-binding proteins then transmit the signal to one of several cascades of protein Ser/Thr kinases that utilize the sequential phosphorylation of kinases to transmit and amplify the signal. These kinase cascades are collectively known as mitogen-activated protein kinase (MAPK) signaling cascades. The best studied of these kinase cascades is the MAPKERR (MAPKp44/p42) signaling cascade. Downstream targets of MAPKERR include p90rsk (p90 ribosomal S6 protein kinase) and the Elk-1 and Stat3 transcription factors. The Jun kinase (MAPKJNK) and MAPKp38 kinase pathways are stress-activated MAPK cascades. The MAPKJNK cascade is activated by inflammatory cytokines as well as by heatshock and UV irradiation. Downstream targets of MAPKJNK include the transcription factors c-Jun and ATF-2. The MAPKp38 pathway is activated by bacterial endotoxins, inflammatory cytokines, and osmotic stress. Downstream targets of MAPKp38 include the transcription factors ATF-2, Max, and CREB. MAPKp38 is also involved in the phosphorylation and activation of heatshock proteins.](image-url)
the steroid/thyroid hormone receptor superfamily of proteins [19,22,23,25–28,31–33,35–37,39–41,43].

The simplified core of a MAPK cascade consists of three protein kinases: a MAP kinase kinase kinase (MAPKKK), a MAP kinase kinase (MAPKK), and MAPK; these kinases phosphorylate each other in sequence. When activated, MAPKKK phosphorylates the MAPK at one or two phosphorylation sites, bringing activation of the MAPKK module. MAPKKs are dual-specificity protein kinases that phosphorylate the MAPK at two phosphorylation sites (almost always a threonine and a tyrosine residue), bringing about activation of the MAPK. The active MAPK can then phosphorylate a variety of target proteins throughout the cytoplasm and nucleus. On the internal regulatory mechanisms, each of the kinases in the MAPK cascade is opposed by one or more phosphoprotein phosphatases. Thus, for an active MAPKK to activate a MAPK, for example, the rate of MAPK phosphorylation must exceed the rate of MAPK dephosphorylation. The activities of these phosphatases are high enough to make the output of an MAPK cascade depend upon the continual presence of a stimulus feeding into the cascade; if this particular stimulus is withdrawn, for instance, the downstream kinases become inactivated within a very short lapse of time [25,27,36,37,39,40].

The best-characterized vertebrate MAPKs fall into three subgroups (see Fig. 2). The first subgroup includes the founding members of the MAPK family, extracellular signal-regulated kinase-1 (ERK1 or MAPKERK1/p44), and ERK2 (or MAPKERK2/p42), and their closest relatives [44–48]. This subgroup is often referred to as ERKs, although some ERK proteins are not in fact members of this subgroup family. The second subgroup is the Jun N-terminal kinases (JNKs), so called because they can activate the Jun transcription factor by phosphorylating two residues near its N-terminus [49–60]. The third subgroup is the p38 MAPKs, so named because of the molecular weight (38 kDa) of the first representative of the subgroup to be discovered [61–68].

Members of both the MAPKJNK and MAPKp38 pathways are also classified as stress-activated protein kinases (SAPKs) because they are activated in response to osmotic shock, UV irradiation, inflammatory cytokines, and other stressful conditions. In all three subgroups, a large number of MAKKKs feed into the activation of a smaller number of MAPKKs and MAPks. The diversity of the MAPKKks thus allows a wide variety of upstream receptors to couple to MAPK cascades (see Fig. 2) [35,69–72]. MAPks and their corresponding substrates are given in Table 1.

### 2.2. MAPK signaling as viewed through receptor and nonreceptor coupled cofactors

The MAPks are a group of closely related families of Ser/Thr kinases involved in regulating growth, differentiation, and cellular responses to stress or inflammatory cytokines. Whereas the MAPKERK pathway essentially regulates growth, proliferation, and differentiation signals, the MAPKJNK/SAPK and MAPKp38 pathways regulate cell responses to environmental stress. The MAPks link signals generated at the cell surface to transcription factors via a cascade of phosphorylation events basically initiated at the level of the membrane by protein tyrosine kinases (PTks), of which two classes exist: the receptor PTks and the nonreceptor PTks. Binding of a ligand to receptor PTk activates the kinase by dimerization and autophosphorylation of specific tyrosine sites on the receptor’s intracellular domain. Among the PTks is

<table>
<thead>
<tr>
<th>Kinase</th>
<th>Substrates</th>
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<tbody>
<tr>
<td>MAPKERK</td>
<td>Elk-1, SAP-1, Mnk-1/2, MAPKAP-K1/p90Rsk, MSK-1</td>
</tr>
<tr>
<td>MAPKJNK/SAPK</td>
<td>c-Jun, ATF-2, Elk-1</td>
</tr>
<tr>
<td>MAPKp38</td>
<td>ATF-2, Elk-1, SAP-1, CHOP, MEF2C, MAPKAP-K2/K3, Mnk-1, MSK-1, PRAK</td>
</tr>
<tr>
<td>MAPKAP-K1/p90Rsk</td>
<td>c-Fos, SRF</td>
</tr>
<tr>
<td>MSK-1</td>
<td>CREB, histone H3 and HMG-14</td>
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For a detailed description, refer to the text.
the Src family of nonreceptor PTKs composed of at least nine members. Most are restricted to defined cellular lines, but three (Src, Fyn, and Yes) are ubiquitously expressed [73]. Like their receptor PTK counterparts, the Srcs are at the upper end of the MAPK cascade and serve as initiators for the transduction of signals generated by receptors at the cell surface. PTK targets contain a 100-amino-acid module SH-2 (Src homology 2) and a 50-amino-acid SH-3 module, which recognizes proline-rich domains [74]. The SH-2 site recognizes tyrosine phosphorylation sites on receptors as well as on nonreceptor proteins [75].

The next step in the activation of the cascade is the binding to, and phosphorylation of, an adaptor protein. Here, two modes have been identified. In one system, phosphotyrosine binds directly to the SH-2 site on Grb2. In the other system, the receptor phosphotyrosine binds to an intermediary adaptor protein Shc, which is subsequently bound to Grb2. Shc, a protein that is widely expressed, is believed to be regulated by translocation from a cytosolic to a plasma membrane location where it recruits Grb2 [76]. Integrins, for example, activate the MAPK ERK pathway by means of an Shc signal. The G-protein-linked thrombin receptor also uses the Shc adapter to transduce mitogenic signaling [77]. The Shc protein also mediates the MAPK response to heat shock response in liver [78]. Shc signaling is utilized by gastrin (in conjunction with Ca$^{2+}$ and PKC) to activate the MAPK ERK1 [79]. Endothelin induces Shc association with Grb2 in glia [80]. Shc binding occurs in response to NGF and EGF in dorsal root ganglion cells [81]. Shc levels are low in the brain; however, other predominantly neural isoforms have been identified: Shc-B, Shc-C, Sck, and n-Shc [82,83]. The latter two are localized exclusively in the brain, with considerable overlap in their expression profiles. The n-Shc variant contains only one high-affinity Grb2-binding site, while Shc has two [84,85]. Grb2 is subsequently complexed with a guanine nuclear exchange factor (GEF).

Two such GEF families have been identified: Sos and Ras-GRF [86,87]. Proteins of the Sos family are ubiquitously expressed, while the Ras-GRF class are expressed primarily in neuronal tissues and are activated by calcium/calmodulin binding as well as by phosphorylation. Ras-GRF-2 is more widely expressed, but is also Ca$^{2+}$-regulated. It appears to be a bifunctional GEF in that it activates the Rho family proteins in addition to Ras. Sos and Ras-GRF activate several of the Ras family proteins, but Ras-GRF uniquely activates a functionally distinct class, R-Ras GTPase. Sos usually complexes with Grb2 to activate Ras. The Ras superfamily is comprised of GTPases that function as molecular switches and exist in the inactive GDP-bound state or the activated GTP-bound form. The GEF proteins activate Ras, while another class of proteins, called GTPase-activating proteins (GAPs), deactivates Ras by reverting it to the GDP-bound state [84,85,87].

The Ras G-proteins mediate the mitogen-induced activation of MAPKs by activating Raf, a family of serine/threonine kinases. Three kinases comprise the Raf family: A-Raf, B-Raf, and Raf-1 [88]. In addition to being activated by the Grb2/Sos/Ras system, Raf also acts in PKC activation of the MAPK ERK cascade. It has been reported that PKC activates C-Raf by phosphorylating serine and that phosphatidate directly activates c-Raf through a diacylglycerol → PKC → phosphatidic acid → c-Raf pathway. Protein kinase A (PKA) has recently been shown to negatively regulate the stimulation of growth factor responses by directly phosphorylating Raf-1 at an undetermined site [89]. MEK1 and MEK2 are the dominant Raf effector proteins, although other Raf substrates have been identified [90]. Activation of MEK1/2 occurs by phosphorylation of serine residues at positions 217 and 221, located in the loop of subdomain VIII. Activation of MAPK ERK1/2 proceeds by phosphorylation of threonine 202 and tyrosine 204.

Deactivation of the MAPKs is an important regulatory feature of the cascade. This is accomplished by phosphatases such as MPK-1/2 PAC-1, which dephosphorylates MAPK ERK in the nucleus, or MPK-3, which dephosphorylates cytosolic MAPK ERK [91]. The MAPKs therefore are a convergence point for a wide variety of extracellular signals. MAPK circuits are a three-tiered module consisting of Raf → MEK → MAPK ERK conveying signals from receptor TKs and G-protein-coupled receptors to transcription factor in the cell nucleus, such as p90-Rsk, c-Myc, Elk-1, and SAP-1. Other targets of MAPK ERK1/2 include myelin basic protein [92] and microtubule-associated protein [93]. Two other signaling cascades are part of the MAPK system. The stress-induced MAPK ERK3 (also
known as HOG in the yeast) mediates inflammatory or stress responses to cytokines, such as TNF, genotoxic agents, osmotic shock, bacterial lipopolysaccharide, and photodamage from ultraviolet light as well as from growth factor withdrawal. The other pathway, MAPK^JNK/SAPK-1, transduces several stress signals including oxidation/DNA damage along with growth and differentiation signals. Both of these pathways are activated by Rac-1 and Cdc-42 signals, which can also activate the MAPK^ERK1/2 system. The three MAPK pathways do not act in isolation but in parallel with limited cross-talk possible (Fig. 3). MKK kinase (MEKK)-3 directly activates both MKK-6 (activator of MAPK^p38) and MKK-7 (activator of MAPK^JNK), while MKK-4 (activator of MAPK^JNK and MAPK^p38) is activated by MEKK-2 and MEKK-3. The MAPK^JNK pathway activates the tumor-suppressor gene, p53, which has recently been demonstrated to counteract Cdc-42 [94].

Multiple isoforms of MAPK^p38 have been identified: p38 (p38-α; SAPK-2a), p38-β (SAPK-2b), p38-β2 (in brain), p38-γ (MAPK^ERK6; SAPK-3), and p38-δ (SAPK-4). The p38-α and p38-β isoforms are ubiquitously expressed. The p38-γ predominates in muscles, while p38-δ is prominent in the lung, kidney, testis, pancreas, and small intestine [95]. Of the various isoforms of MAPK^p38, only p38-α and p38-β are sensitive to inhibition by SB-203580.

Several activation routes converge on p38. The Rac-1 and Cdc-42 signals activate a family of mixed lineage kinases (MLKs), which further activate MAP kinase kinases (MKKs). Rac-1 activates MEKK-1 and

![Fig. 3. An overview of the complex network of MAPK signaling pathways and their interactions and bifurcations. The sign (−) denotes inhibition as (−); the solid arrows indicate activation (stimulation); P, phosphorylation. NB: Phosphatide indicates a lipid or phospholipid intermediate relaying signals from PKC to C-Raf in the pathway. Abbreviations are listed and defined in the text.](image-url)
MEKK-2, which subsequently activate MKK-4 that phosphorylates MAPK
p38. MKK-4 mRNA is widely expressed in murine tissues, but is most abundant in the muscles and brain [96]. Another route for MAPK
p38 activation is through MKK-6 (MAPKK-6; MKK-6). MKK-6 (and its variant MKK-6b) activates p38-α, p38-β2, and p38-γ, but p38-δ is not activated by MKK-6. This pathway is activated by Rac-1 and Cdc42 signals via MLKs and also transduces TGFβ signals from the TGFβRI receptor complex via Tak-1, as well as by Ask-1, a transducer of signals from FasL, inflammatory cytokines, or stressful insults such as UV irradiation or apoptotic signals. MKK-3 activates p38-α, p38-β, p38-γ, and p38-δ [95,96]. MKK-6 activates only the p38-β2 isoform. Additionally, MKK-7, like MKK-6 and MKK-4, phosphorylates p38-α at the tyrosine of a Thr–Gly–Tyr motif, but does not phosphorylate the other MAPK
p38 isoforms (see Fig. 3).

The individual MAPK
p38 isoforms are activated by specific stimuli and in turn act on a wide range of substrates. The p38-α isoform is activated by cellular stress, TNF-α, IL-1β, fMLP, PAF lipopolysaccharides, anisomycin, IL-3, and CD-40l [96,97]. Its substrates are: ATF-2; MAPKAPs 2, 3, and 5; Sap1a; CHOP-1 Elk-1 MEF-2C; MSK-1; MBP; PRAK; P47phox; and MNK-1 [95–98]. The p38-β2 isoform is activated by cellular stress, TNF-α, IL-1β, UV, NaCl, and anisomycin, and its substrates are: ATF-2, MAPKAPs 2 and 3, PRAK, and Sap-1a. The p38-γ and p38-δ isoforms share similarities in activation responses and substrates. Both are activated by osmotic changes PMA IL-1β and TNF-α, but p38-δ also responds to cellular stress. Both kinases act upon the same set of substrates: ATF-2, statmhin, Elk-1, Sap-1a, and MBP. Other substrates for MAPK
p38 are MSK-1 and the cytoplasmic Tau proteins [98–100].

The transcription factor c-Jun mediates cell stress responses [100,101]. The activity of c-Jun is regulated by phosphorylation of its N-terminal region. The MAPK
JNK is encoded by three genes jnk1 and jnk2, which are ubiquitously expressed, and jnk3, which is restricted to the brain, heart, and testis. MAPK
JNK exists in a series of alternatively 3’-spliced variants. Alternative splicing of MAPK
JNK1 and MAPK
JNK2 results in two classes by size: 46 kDa (JNK-1b, JNK-2b) and 55 kDa (JNK-1a, JNK-2a). A second splicing alternative involving exons in the kinase subdomains IX and X yields additional variants JNK1-a1, JNK1-a2, JNK1-b1, JNK1-b2, JNK2-a1, JNK2-a2, JNK2-b1, and JNK2-b2. The region producing these different variants is a 23-amino-acid segment between positions 208 and 230 in the protein’s C-terminal region abutting the catalytic domain of the enzyme. Alternative MAPK
JNK3 splicing yields only a pair of variants 49 kDa (JNK-3b) and 57 kDa (JNK-3a) [101].

Activation of MAPK
JNK1 and MAPK
JNK2 requires phosphorylation of threonine at position 183 and tyrosine at position 185, while for MAPK
JNK3 activation, threonine at position 221 and tyrosine at position 223 are phosphorylated. MKK-4 and MKK-7 synergistically activate MAPK
JNK by phosphorylating at Thr-183, Tyr-185, Thr-404, and Ser-407. MKK-4 preferably phosphorylates Tyr-185 on the Thr–Pro–Tyr motif on subdomain VII [101]. MKK-7 encodes a reading frame of 347 amino acids with 11 kinase subdomains [102]. MKK-7 exists in two variants: MKK-7α, which preferentially phosphorylates Thr-183, and MKK-7β, which is equally specific for Thr-183 and Tyr-185. MKK-7β is hundredfold more efficient at phosphorylating all three MAPK
JNK isoforms than MKK-7α [103]. Thr-404 and Ser-407 are additional phosphorylation sites for MKK-7 action. MEKK-2 and -3 activate MKK-7 and MKK-4, two alternative routes leading to MAPK
JNK activation.

Recently, a new ubiquitously expressed 898-residue kinase, DPK, has been described, which shows homology with MEKKs 1–5 and p21-activated kinase (PAK), but no GTPase binding. DPK activates MAPK
ERK1/2 and MAPK
JNK, but not MAPK
p38 [104]. Like in the MAPK
p38 pathway, Rac and Cdc42 initiate the cascade leading to MAPK
JNK activation, in response to cytokines or stressful stimuli. The MAPK
JNK system is also activated by ceramide via Tak-1 and by TNF-α via Ask-1 signals, both activating MKK-4 and MKK-7 [100]. Cadmium activation of MAPK
JNK is mediated by MKK-7 [100,101].

Negative regulators of MAPK
JNK have also been described. In the central nervous system, the excitotoxin kainic acid downregulates MAPK
JNK1. Glutathione S-transferase Pr (GSTp) is an endogenous inhibitor of MAPK
JNK. GSTp is associated with MAPK
JNK under baseline conditions and its dissociation from MAPK
JNK leads to disinhibition. Another class of negative regulator is the MAPK phosphatases (MKP), which antagonize MAPK
JNK by dephosphor-
ylating MAPKJNK as well as its substrate. There are three isoforms of MKP: MKP-1, which recognizes MAPKERK, MAPKp38, and MAPKJNK; MKP-2, which recognizes MAPKERK and MAPKJNK; and MKP-3, which is specific for MAPKERK [100]. The heat shock protein, Hsp-72, is also an inhibitor of MAPKJNK [105].

The translocation of activated MAPKJNK is regulated by a class of proteins called JNK-interacting proteins (JIPs). Several JIP isoforms have been identified: JIP-1, expressed in the brain, kidney, and elsewhere; JIP-2 and JIP-2a; and JIP-3, expressed exclusively in the brain. JIP-1 contains a phosphotyrosine-interacting domain as well as an SH-3 domain. JIP inhibits the action of MAPKJNK by binding and retaining MAPKJNK in the cytoplasm [101,106]. MAPKJNK acts upon both nuclear and cytoplasmic substrates, but does not have as wide a repertoire as MAPKp38. MAPKJNK activates the transcription factors c-Jun, ATF-2, and Elk-1, while NFAT-4 is inactivated. MAPKJNK also inhibits the transcriptional effect of glucocorticoid receptor; the antiapoptosis cofactor, Bcl-2, is also deactivated. MAPKJNK phosphorylates Tau protein, neurofilaments, and MADD [100,107]. Additionally, MAPKJNK regulates p53, which in turn regulates Cdc-42 activity as well as operates in apoptosis. These complex MAPK cascades and networks are schematically summarized in Fig. 3.

2.3. MAPK signaling and redox-mediated regulation of kinases/phosphatases

As discussed above, MAPKs are key signal-transducing enzymes that are activated by a wide range of extracellular stimuli. Although regulation of MAPKs by a phosphorylation cascade has long been recognized as significant, their inactivation through the action of specific phosphatases has been less studied. An emerging family of structurally distinct dual-specificity serine, threonine, and tyrosine phosphatases that act on MAPKs consists of 10 members in mammals, and members have been found in animals, plants, and yeast [108].

An early role of oxidants in regulating kinases/phosphatases involved with MAPK induction/inhibition was reported in neutrophils [109]. It has been demonstrated that reactive oxygen species (ROS) were produced by the oxidase regulate tyrosine phosphorylation, possibly by alterations in the cellular redox state. For example, immunoprecipitation of MAPK indicated that a 42- to 44-kDa polypeptide was tyrosine-phosphorylated in response to treatment of cells, either with the oxidizing agent diamide or with H2O2 in cells where catalase was inhibited. Furthermore, exposure of cells to oxidants caused a significant increase in the activity of MEK, as determined by an in vitro kinase assay using recombinant catalytically inactive glutathione S-transferase MAPK as the substrate. Additionally, oxidant treatment of cells resulted in inhibition of the activity of CD45, a protein tyrosine phosphatase known to dephosphorylate and inactivate MAPK, thereby concluding that oxidant treatment of neutrophils can activate MAPK by stimulating its tyrosine and, presumably, threonine phosphorylation via MEK activation, a response that may be potentiated by inhibition of MAPK dephosphorylation by phosphatases such as CD45 [109].

The aforementioned observations were also reaffirmed in muscle cells [110]. Vascular smooth muscle cell growth, as measured by [3H]thymidine incorporation, was stimulated by H2O2 and the naphthoquinolinedione LY83583. There was an increase in MAPK activity by LY83583 but not by H2O2. In addition, activation of MAPK by LY83583 was PKC-dependent. Of note, expression of MAP kinase phosphatase-1 (MKP-1), a transcriptionally regulated redox-sensitive protein tyrosine/threonine phosphatase, showed that although H2O2 induced MKP-1 mRNA to a greater extent than did LY83583, the increased MKP-1 expression could not explain the inability of H2O2 to stimulate MAPK because mRNA levels were not detected until an hour later, thereby indicating that additional signal events are required for the mitogenic effects of H2O2 [110]. Moreover, the thiol-depleting agents, phenylarsine oxide (a tyrosine phosphatase inhibitor) and N-ethylmaleimide, were reported to inhibit the phorbol ester-induced PKC activation in vascular smooth muscle cells [111]. In addition, sodium orthovanadate, also a protein tyrosine phosphatase inhibitor, could neither activate nor inhibit PKC, suggesting that oxidation of the cellular thiols inhibits PKC and activates MAPK, indicating that the activation of MAPK is independent of PKC.
In mesangial cells, redox- and oxidant-dependent assessment of MAPK identified a rapid and delayed phase of activation. For instance, rapid and late MAPK activations were attenuated by the redox-modulating agent, N-acetylcysteine [112]. Specifically, late-phase activation coincided with endogenous nitric oxide (NO) generation and in turn was suppressed by the NO synthase-blocking compound diphenylennidonium or nitroarginine methyl ester. Of particular interest, late and persistent MAPK activation, induced by NO donors or endogenously generated NO, was found in association with inhibition of phosphatase activity. Moreover, in vitro dephosphorylation of activated and immunoprecipitated p42/p44 by cytosolic phosphatases was sensitive to the readdition of NO and was found to be inhibited in the cytosol of S-nitrosogluthathione-stimulated cells. Conclusively, NO affects MAPK ERK twofold: rapid activation is cGMP-mediated, whereas late activation is transmitted via inhibition of tyrosine dephosphorylation [112]. In concert, it has been demonstrated that a redox-sensitive protein phosphatase activity regulates the phosphorylation state of MAPKp38 in primary astrocyte culture, suggesting that ROS are used as second-messenger substances that activate MAPKp38 in part via the transient inactivation of regulatory protein phosphatases [113]. On the particular mechanism involved in redox-mediated regulation of MAPKs, it has been suggested that the differential interaction of the tyrosine phosphatases PTP-SL, STEP, and HePTP with MAPK ERK and MAPKp38α could be determined by a kinase specificity sequence and influenced by reducing agents [114], indicating that intracellular redox conditions could modulate the activity and subcellular location of MAPKs by controlling their association with their regulatory PTPs. A closely related mechanism was reported for cell adhesion. It was indicated that ROS are essential mediators of cell adhesion; specifically, the oxidative inhibition of a tyrosine phosphatase is required for cell adhesion. This signaling network unraveled a redox circuitry whereby, upon cell adhesion, oxidative inhibition of a protein tyrosine phosphatase promotes the phosphorylation/activation and the downstream signaling MAPKs and, as a final event, cell adhesion and spreading onto fibronectin [115].

3. Hypoxia-mediated regulation of MAPK signaling pathways

3.1. Hypoxia and oxygen-sensing mechanisms

How do organisms sense the level of oxygen in the environment/microenvironment and respond appropriately when oxygen level decreases (a condition termed hypoxia) [9,10,19,112,116,117]? The expression of genes is predominantly determined by conditions of the cell microenvironment. Prime examples of such regulation are found in embryonic development of all multicellular organisms. The naturally occurring regulating agents, for example, interact with specific receptors, which subsequently transduce a signal onto the nucleus for the regulation of gene expression and activation. The putative oxygen sensor responds to dynamic variation in pO2 such as those occurring during the birth transition period. Upon ligand binding, this presumably membrane-bound receptor transduces intracellular chemical/redox signals that relay messages for the regulation of gene expression, a phenomenon mainly involving the activation of transcription factors, ion channels, and chemoreceptors (Fig. 4).

Oxygen sensing and the underlying molecular stratagems have been the focus of experimental investigations trying to find an answer to the question: “What is the identity of the oxygen sensor?” [5–8]. The original proposed molecular mechanism underlying oxygen sensing in mammalian cells involves an oxygen sensor that is a heme protein. Studies on erythropoietin (EPO), a glycoprotein hormone required for the proliferation and differentiation of erythroid cells, demonstrate that EPO production is enhanced under hypoxic conditions. Furthermore, the induction of EPO expression by transition metals such as cobalt (Co2+) and nickel (Ni2+) supports the hypothesis that the oxygen sensor for the induction of this glycoprotein is a heme protein and that these metal atoms can substitute for the iron atom within the heme moiety [5,6,118,119].

Further evidence supporting the notion that the oxygen sensor is a heme protein came with additional studies that utilized carbon monoxide (CO); CO can noncovalently bind to ferrous (Fe2+) heme groups in hemoglobin, myoglobin, cytochromes, and other heme proteins [5,6], where its ligation state is struc-
turally identical to that of oxygen. It was subsequently proposed that the effect of CO on oxygen sensing might occur via locking of the sensor in an oxy-
conformation, which could involve a multisubunit mechanism [118–120].

In addition to the aforementioned models for oxygen sensing, certain pharmacological studies, led by Fandrey et al. [121], suggest that the oxygen sensor might involve a microsomal mixed function oxidase. Based on these studies, it was proposed that oxygen sensing for EPO involves an interaction between cytochrome P450 and cytochrome P450 reductase, thereby allowing the conversion of molecular oxygen to superoxide anion (O$_2^-$) and H$_2$O$_2$ radicals [5–8,119–122]. Acker [123] has provided support for the central role of an oxidase in oxygen sensing based on spectroscopic evidence. It was reported that b-cytochrome functions as a NAD(P)H oxidase, converting oxygen to O$_2^-$. The enzymatic complex in mammalian cells is membrane-bound and transduces the conversion of molecular oxygen to ROS, according to the following equations:

\[
\text{CytFe}^{2+} + O_2 \rightarrow \text{CytFe}^{2+}O_2
\]

\[
\text{CytFe}^{2+}O_2 \rightarrow \text{CytFe}^{3+} + O_2^-
\]

\[
\text{CytFe}^{3+} + \text{NAD(P)}H \rightarrow \text{CytFe}^{2+} + \text{NAD(P)}^+
\]

A resurgence of interest in mitochondrial physiology has recently developed as a result of new experimental data demonstrating that mitochondria function as important participants in a diverse collection of novel intracellular signaling pathways. Further experiments showed a potential involvement of the mito-

Fig. 4. Oxygen-sensing mechanisms during hypoxia involve the membrane-bound NADPH oxidase, the mitochondria–cytochrome complex system (A), and chemoreceptors (B), such as K$^+$ channels. This ultimately leads to sensory recognition and activity upregulation.
chondria in oxygen sensing [124]. For instance, a spectroscopic photolysis with monochromatic light has identified a CO-binding heme protein falling within the spectrum of the mitochondrial cytochrome $a_3$ [125]. It was consequently proposed that this heme protein, presumably located on the plasma membrane, has a low affinity for oxygen and a relatively high affinity for CO. The same model predicted that another heme protein in the mitochondria has a relatively higher affinity for oxygen and a lower affinity for CO. The biochemical reaction, which was proposed as an alternative way of regenerating ferroheme in the oxygen sensor, is given below:

$$CO + 2Fe^{3+} + H_2O \rightarrow CO_2 + 2Fe^{2+} + 2H^+$$

These aforementioned observations pertaining to the mitochondrion as a possible oxygen sensor were unequivocally supported by novel studies recently reported. Cardiomyocytes are known to suppress contraction and oxygen consumption during hypoxia. Cytochrome oxidase undergoes a decrease in $V_{max}$ during hypoxia, which could alter mitochondrial redox status and increase the generation of ROS. Duranteau et al. [126] tested whether ROS generated by mitochondria act as second messengers in the signaling pathway linking the detection of oxygen with the functional response. In this respect, contracting cardiomyocytes were superfused under controlled oxygen conditions, while fluorescence imaging of 2,7-dichlorofluorescein (DCF) was used to assess ROS generation. Compared with normoxia, graded increases in DCF fluorescence were seen during hypoxia. In addition, the antioxidants 2-mercaptopropionyl glycine and 1,10-phenanthroline attenuated these increases and abolished the inhibition of contraction. Superfusion of normoxic cells with $H_2O_2$ mimicked the effects of hypoxia by eliciting decreases in contraction that were reversible. To test the role of cytochrome oxidase, sodium azide was added during normoxia to reduce the $V_{max}$ of the enzyme. It was observed that azide produced graded increases in ROS signaling, accompanied by graded decreases in contraction that were reversible, demonstrating that mitochondria respond to graded hypoxia by increasing the generation of ROS and suggesting that cytochrome oxidase may contribute to this oxygen-sensing mechanism [126].

The same group also reported that mitochondrial ROS trigger hypoxia-induced transcription. Chandel et al. [127] tested whether mitochondria act as oxygen sensors during hypoxia, and whether hypoxia and CO activate transcription by increasing the generation of ROS. Results showed that: (i) wild-type Hep3B cells increased ROS generation during hypoxia or CoCl$_2$ incubation; (ii) Hep3B cells depleted of mitochondrial DNA ($\rho^0$ cells) failed to respire; failed to activate mRNA for EPO, glycolytic enzymes, or vascular endothelial growth factor (VEGF) during hypoxia; and failed to increase ROS generation during hypoxia; (iii) $\rho^0$ cells increased ROS generation in response to CoCl$_2$ and retained the ability to induce expression of these genes; and (iv) the antioxidants pyrrolidine dithiocarbamate (PDTC) and ebselen, a glutathione (GSH) peroxidase mimic, abolished transcriptional activation of these genes during hypoxia or CoCl$_2$ in wild-type cells and abolished the response to CoCl$_2$ in $\rho^0$ cells [127]. It was proposed that hypoxia activates transcription via a mitochondria-dependent signaling process involving increased ROS, whereas CoCl$_2$ activates transcription by stimulating ROS generation via a mitochondria-independent mechanism [126–129].

In another interesting observation, Chandel et al. reported that mitochondrial ROS play a major role in HIF-1$\alpha$ regulation. In this respect, it was observed that hypoxia increased mitochondrial ROS generation at complex III, which caused the accumulation of HIF-1$\alpha$ protein responsible for initiating expression of a luciferase reporter construct under the control of a hypoxic response element [130]. Of note, this response was lost in cells depleted of mitochondrial DNA. Furthermore, overexpression of catalase abolished hypoxic response element luciferase expression during hypoxia. In addition, exogenous $H_2O_2$ stabilized HIF-1$\alpha$ protein during normoxia and activated luciferase expression in wild-type and $\rho^0$ cells. In fact, isolated mitochondria increased ROS generation during hypoxia, indicating that mitochondria-derived ROS are both required and sufficient to initiate HIF-1$\alpha$ stabilization during hypoxia, thereby implicating this transcription factor as a possible oxygen sensor (see below).

A nonmitochondrial oxygen sensor has, however, been recently proposed. Ehleben et al. applied biophysical methods, such as light absorption spectropho-
tometry of cytochromes, determination of NAD(P)H-dependent O$_2$ formation, and localization of -OH by three-dimensional (3D) confocal laser scanning microscopy, to reveal putative members of the oxygen-sensing signal pathway leading to enhanced gene expression under hypoxia [131,132]. A cell membrane-localized nonmitochondrial cytochrome $b_{558}$ seemed to be involved as an oxygen sensor in the hepatoma cell line HepG2 in cooperation with the mitochondrial cytochrome $b_{563}$, probably probing additionally metabolic changes. The hydroxyl radical, a putative second messenger of the oxygen-sensing pathway generated by a Fenton reaction, could be visualized in the perinuclear space of the three human cell lines used. Substances like cobalt or the iron chelator desferrioxamine, which have been applied in HepG2 cells to mimic hypoxia-induced gene expression, interact on various sides of the oxygen-sensing pathway, confirming the importance of b-type cytochromes and the Fenton reaction.

NADPH oxidase isoforms, with different gp91phox subunits as well as an unusual cytochrome $aa_3$ with a heme $a/a_3$ relationship of 9:91, were discussed as putative oxygen sensor proteins influencing gene expression and ion channel conductivity [133]. ROS are believed to be important second messengers of the oxygen-sensing signal cascade determining the stability of transcription factors or the gating of ion channels. The formation of ROS by a perinuclear Fenton reaction was imaged by one- and two-photon confocal microscopy revealing mitochondrial and nonmitochondrial generation. In reference to the aforementioned observation, some recent concepts on oxygen-sensing mechanisms at the carotid body chemoreceptors were highlighted [134]. Most available evidence suggested that glomus (type I) cells are the initial sites of transduction, and they release transmitters in response to hypoxia, which in turn depolarize the nearby afferent nerve ending, leading to an increase in sensory discharge.

Two main hypotheses have been advanced to explain the initiation of the transduction process that triggers transmitter release. One hypothesis assumed that a biochemical event associated with a heme protein triggers the transduction cascade. Supporting this idea, it has been shown that hypoxia might affect mitochondrial cytochromes. In addition, there was a body of evidence implicating nonmitochondrial enzymes such as NADPH oxidases, NO synthases, and heme oxygenases located in glomus cells [134]. These proteins could contribute to transduction via generation of ROS, NO, and/or CO.

The other hypothesis suggested that a K$^+$ channel protein is the oxygen sensor, and that inhibition of this channel and the ensuing depolarization is the initial event in transduction, as indicated by Peers and Kemp [135]. Several oxygen-sensitive K$^+$ channels have been identified. However, their roles in the initiation of the transduction cascade and/or cell excitability remain unclear. In addition, recent studies indicated that molecular oxygen and a variety of neurotransmitters might also modulate Ca$^{2+}$ channels [134]. Most importantly, it is possible that the carotid body response to oxygen requires multiple sensors, and they work together to shape the overall sensory response of the carotid body over a wide range of arterial oxygen tensions.

The hypothesis that there exists a specific oxygen sensor(s), which relay(s) chemical signals intracellularly, is therefore consistent with the notion that there is a unifying mechanism involved in transducing dynamic changes in pO$_2$ to the nucleus [5–8]. In response to ΔpO$_2$, there is a coordinate expression of genes needed to confer appropriate responses to hypoxia or hyperoxia. The regulation of physiologically important oxygen-responsive and redox-sensitive genes would, therefore, dictate well-controlled responses of the cell within a challenging environment and necessarily would determine the specificity of cellular adaptation.

3.2. Hypoxia and MAPK signaling

In 1997, Seko et al. [136] directly and unequivocally reported that both hypoxia and hypoxia/reoxygenation rapidly activate upstream Src family TyrKs and p21ras. This was followed by the sequential activation of MAPKKK activity of Raf-1, MAPKK, MAPKs including MAPKi ERK1/2, and S6 kinase (p90-rsk). Furthermore, it was demonstrated that hypoxia and hypoxia/reoxygenation could cause rapid activation of stress-activated MAPK signaling cascades involving p65-PAK, MAPK$^{p38}$, and SAPK. These stimuli also caused the phosphorylation of activating the downstream transcription factor (ATF)-2 [137]. Because p65-PAK is known to be upstream of MAPK$^{p38}$ and
also a target of p21rac-1, which belongs to the Rho subfamily of p21ras-related small GTP-binding proteins, these results suggested that two different stress-activated MAPK pathways distinct from the classical MAPK pathway were activated in response to hypoxia and hypoxia/reoxygenation.

In concert, Yu et al. [138] reported that cardiomyocytes subjected to brief episodes of hypoxia possess a resistance to serious damaging effects exerted by a subsequent long-time hypoxia on these cells, a condition that was termed hypoxic preconditioning (PC). On a model of hypoxia/reoxygenation of cultured neonatal rabbit cardiomyocytes, the changes of MAPK and RSK activity were recorded. It was found that intracellular total MAPK and nuclear MAPK, after a period of reoxygenation-preceded hypoxia, were increased [138]. Moreover, intracellular RSK activity increased by hypoxia/reoxygenation. Phosphatase-1 (Pphase-1) inhibitor (okadaic acid, or OA) augmented the increase of MAPK and RSK activity induced by hypoxia/reoxygenation. However, TyrK inhibitor (genistein), PKC inhibitor (H7), and preincubation of cardiomyocytes with PKC activator PMA all reduced MAPK activation by hypoxia/reoxygenation. In addition, PKA inhibitor (H89), Ca\(^{2+}\)/calmodulin (CaM)-dependent protein kinase (PKM) inhibitor (W7), or Pphase-2a inhibitor (OA) had no effect on MAPK and RSK activity, thus indicating that the activation of MAPK and RSK activity during hypoxia/reoxygenation might require the participation of PKC, TyrK, and Pphase-1, while PKA, PKM, and Pphase-2a were not involved.

Further elaborating on the mechanisms involved in hypoxia-mediated regulation of MAPK signaling, Mizukami et al. [139] recently reported that PKC-\(\alpha\), an atypical PKC isoform mainly expressed in rat heart, can act as an upstream kinase of MAPK during ischemic hypoxia and reoxygenation. Immunocytochemical observations showed PKC-\(\alpha\) staining in the nucleus during ischemic hypoxia and reoxygenation when phosphorylated MAPK was also detected in the nucleus. This nuclear localization of PKC-\(\alpha\) was inhibited by treatment with Wortmannin. This was supported by the inhibition of MAPK phosphorylation by another blocker of phosphoinositide 3-kinase, LY294002. Moreover, an upstream kinase of MAPK, MEK1/2, was significantly phosphorylated after reoxygenation (observed mainly in the nucleus), whereas it was present in the cytoplasm in serum stimulation. PKC inhibitors and phosphoinositide 3-kinase inhibitors, as observed in the case of MAPK phosphorylation, blocked the phosphorylation of MEK, indicating that PKC-\(\alpha\), which is activated by phosphoinositide 3-kinase, might induce MAPK activation through MEK in the nucleus during reoxygenation after ischemic hypoxia.

In parallel, exposure to moderate hypoxia (5% \(O_2\)) was found to progressively stimulate the phosphorylation and activation of different isoforms of MAPK\(^{p38}\), such as MAPK\(^{p38-\gamma}\), in particular, and also MAPK\(^{p38-\alpha}\). In contrast, hypoxia had no effect on the enzyme activity of MAPK\(^{p38-\beta}\), MAPK\(^{p38-\delta}\), MAPK\(^{p38-\delta}\), or even on MAPK\(^{JNK}\) [139]. Prolonged hypoxia also induced the phosphorylation and activation of MAPK\(^{ERK1/2}\), although this activation was modest when compared to NGF and UV-induced activation [140]. It was also shown that the activation of MAPK\(^{p38-\gamma}\) during hypoxia required calcium (\(Ca^{2+}\)), as treatment with \(Ca^{2+}\)-free media or the CaM antagonist, W13, blocked the activation of MAPK\(^{p38-\gamma}\). These studies demonstrated that an extremely typical physiological stress could cause selective activation of specific elements of the SAPKs and MAPKs, and identify \(Ca^{2+}\)/CaM as a critical upstream activator. The role of hypoxia in mediating MAPK signaling is schematized in Fig. 5.

**Fig. 5.** Hypoxia-mediated regulation of MAPK signaling pathways. The role of the different MAPK signaling pathways in regulating cellular responses, including cell survival and death, in response to incoming signals such as oxygen deprivation (hypoxia).
4. Hypoxia-mediated regulation of transcription factors and gene transcription: the role of MAPK-related signaling pathways

4.1. MAPK-mediated regulation of HIF

4.1.1. HIF and oxygen sensing—an overview

In order to maintain oxygen homeostasis, a process that is, of course, essential for survival, pO₂ delivery to the mitochondrial electron transport chain must be tightly maintained within a narrow physiological range [5–8]. However, this system may fail with subsequent induction of hypoxia, resulting in either a failure to generate sufficient ATP to sustain metabolic activities, or a hyperoxic condition that contributes to the generation of ROS, which, in excess, could be cytotoxic and often cytocidal. Adaptive responses to hypoxia involve the regulation of gene expression by HIF-1α, whose expression, stability, and transcriptional activity increase exponentially on lowering pO₂ [141–143].

HIF-1α is a mammalian transcription factor expressed uniquely in response to physiologically relevant hypoxic conditions [5–8,141–143]. Studies of the EPO gene led to the identification of a cis-acting hypoxia response element (HRE) in the 3′-flanking region. HIF-1 was identified as a hypoxia-inducible HRE-binding activity. The HIF-1 binding site was subsequently used for purification of the HIF-1α and HIF-1β subunits by DNA affinity chromatography. Both HIF-1 subunits are basic helix–loop–helix (bHLH)–PAS proteins: HIF-1α is a novel protein; HIF-1β is identical to the aryl hydrocarbon receptor nuclear translocator (ARNT) protein. HIF-1α DNA-binding activity and HIF-1α protein expression are rapidly induced by hypoxia and the magnitude of the response is inversely related to pO₂ [142–145].

In hypoxia, multiple systemic responses are induced, including angiogenesis, erythropoiesis, and glycolysis. HREs containing functionally essential HIF-1 binding sites are identified in genes encoding VEGF, glucose transporter-1 (GLUT-1), and the glycolytic enzymes aldolase A, enolase-1 (ENO-1), lactate dehydrogenase A (LDH-A), and phosphoglycerate kinase-1 [5–8]. HIF-1α is an important mediator for increasing the efficiency of oxygen delivery through EPO and VEGF. A well-controlled process of adaptation parallels this with decreased oxygen availability through the expression and activation of glucose transporters and glycolytic enzymes. Of note, EPO is responsible for increasing blood oxygen-carrying capacity by stimulating erythropoiesis, VEGF is a transcriptional regulator of vascularization and glycolytic transporters, and enzymes increase the efficiency of anaerobic generation of ATP.

HIF-1α has also been shown to activate transcription of genes encoding inducible nitric oxide synthase (iNOS) and heme oxygenase-1 (HO-1)—which are responsible for the synthesis of the vasoactive molecules NO and CO, respectively—as well as transferrin—which, like EPO, is essential for erythropoiesis. Each of these genes contains an HRE sequence of <100 bp that includes one or more HIF-1 binding sites containing the core sequence 5′-RCGTG-3′ [5–8]. It is expected that any reduction of tissue oxygenation in vivo and in vitro would therefore provide a mechanistic stimulus for a graded and adaptive response mediated by HIF-1α. Hypoxia signal transduction is schematized in Fig. 6.

Several of the major molecular mechanisms that regulate HIF-1 have recently emerged to shed a
thorough light on the role of this transcription factor in oxygen sensing [144,145]. The von Hippel–Lindau protein (pVHL) has emerged as a key factor in cellular responses to oxygen availability, being required for the oxygen-dependent proteolysis of the α-subunits of HIF (Fig. 7). Mutations in VHL cause a hereditary cancer syndrome associated with dysregulated angiogenesis and upregulation of hypoxia-inducible genes [145]. Recently, Lee et al. [142], Salceda and Caro [143], Zhu and Bunn [144], and Maxwell and Ratcliffe [145] unequivocally elaborated on the mechanisms underlying these processes and showed that extracts from VHL-deficient renal carcinoma cells have a defect in HIF-1α ubiquitination activity, which was complemented by exogenous pVHL. This defect was specific for HIF-1α among a range of substrates tested.

Furthermore, HIF-1α subunits were the only pVHL-associated proteosomal substrates identified by comparison of metabolically labeled anti-pVHL immunoprecipitates with proteosomally inhibited cells and normal cells. Analysis of pVHL/HIF-1α interactions defined short sequences of conserved residues within the internal transactivation domains of HIF-1α molecules sufficient for recognition by pVHL. In contrast, while full-length pVHL and the p19 variant interacted with HIF-1α, the association was abrogated by further N-terminal and C-terminal truncations. The interaction was also disrupted by tumor-associated mutations in the β-domain of pVHL and loss of interaction was associated with defective HIF-1α ubiquitination and regulation, defining a mechanism by which these mutations generate a constitutively hypoxic pattern of gene expression promoting angiogenesis [145–148]. These findings clearly indicate that pVHL regulates HIF-1α proteolysis by acting as the recognition component of a ubiquitin ligase complex and supports a model in which its β-domain interacts with short recognition sequences in the α-subunits.

Moreover, in oxygenated and iron-replete cells, HIF-1α subunits were rapidly destroyed by a mechanism that involved ubiquitination by the pVHL E3 ligase complex [149]. This process was suppressed by hypoxia and iron chelation, allowing transcriptional activation. Jaakkola et al. [149] recently indicated that the interaction between human pVHL and a specific domain of the HIF-1α subunit is regulated through hydroxylation of a proline residue (HIF-1α P564) by an enzyme termed by the authors as HIF-α prolyl-hydroxylase (HIF-PH or HPH). An absolute requirement for oxygen as a cosubstrate and iron as a cofactor suggested that HIF-PH functions directly as a cellular oxygen sensor. Furthermore, Masson et al. [150] recently identified two independent regions within the HIF-α oxygen-dependent degradation domain (ODDD), which are targeted for ubiquitination by VHLE3 in a manner dependent upon prolyl hydroxylation (Fig. 8). In a series of in vitro and in vivo assays, Masson et al. demonstrated the independent and nonredundant operation of each site in the regulation of the HIF system. Both sites contain a common core motif, but differ both in overall sequence and conditions under which they bind to the VHLE3 ligase complex [150]. The definition of two independent destruction domains implicated a more complex system of pVHL–HIF–α interactions, but reinforced the role of prolyl hydroxylation as an oxygen-dependent destruction signal.

These mechanisms were also reported in lower invertebrates as potential pathways for HIF oxygen sensing. For instance, Epstein et al. [151] defined a conserved HIF–VHL–prolyl hydroxylase pathway in Caenorhabditis elegans and used a genetic approach to identify EGL-9 as a dioxygenase that regulates HIF by prolyl hydroxylation. In mammalian cells, it was shown that the HIF-prolyl hydroxylases were repre-

Fig. 7. Potential oxygen-sensing mechanisms and the role of the transcription factor HIF. This schematic shows the role of von Hippel–Lindau (VHL) tumor-suppressor protein in mediating the regulation of HIF.
sented by a series of isoforms bearing a conserved 2-histidine-1-carboxylate–iron coordination motif at the catalytic site. Direct modulation of recombinant enzyme activity by graded hypoxia, iron chelation, and cobaltous ions mirrored the characteristics of HIF induction in vivo, thereby fulfilling requirements for these enzymes as oxygen sensors that regulate this transcription factor[152–155]. Similarly, Bruick and McKnight [156] reported that the inappropriate accumulation of HIF caused by forced expression of the HIF-1α subunit under normoxic conditions was attenuated by coexpression of HPH. Suppression of HPH in cultured Drosophila melanogaster cells by RNA interference resulted in elevated expression of a hypoxia-inducible gene (LDH; encoding lactate dehydrogenase) under normoxic conditions, indicating that HPH is an essential component of the pathway through which cells sense oxygen. In complement with the aforementioned observations, Lando et al. [157] demonstrated that the hypoxic induction of the COOH-terminal transactivation domain (CAD) of HIF occurs through abrogation of hydroxylation of a conserved asparagine (Asn) in the CAD. Inhibitors of Fe2+ - and 2-oxoglutarate-dependent dioxygenases prevented hydroxylation of the Asn, thus allowing the CAD to interact with the p300 transcription coactivator. Replacement of the conserved Asn by alanine (Ala) resulted in constitutive p300 interaction and strong transcriptional activity. Moreover, the full induction of HIF, therefore, possibly relies on the abrogation of both proline (Pro) and Asn hydroxylation, which, during normoxia, occurs at the degradation and COOH-terminal transactivation domains, respectively.

Recently, an oxygen-sensitive cousin of HIF-1 has been identified, characterized, and cloned. Hypoxia-inducible factors (HIF-1, HIF-2, and HIF-3) are closely related protein complexes that are oxygen-responsive. The cDNAs of three HIF α-subunits were cloned from RNA of primary rat hepatocytes by reverse transcriptase polymerase chain reaction [158]. All three cDNAs encoded functionally active proteins of 825, 874, and 662 amino acids, respectively. After transfection, they were able to activate the luciferase activity of a luciferase gene construct containing three HIF-responsive elements. The mRNAs of the rat HIF α-subunits were expressed predominantly in the peri-venous zone of rat liver tissues; the nuclear HIF-α proteins, however, did not appear to be zonated [158]. HIFs locate to HIF-binding sites (HBS) within the HREs of oxygen-regulated genes [159,160]. Whereas HIF-1α is, generally, expressed ubiquitously, HIF-2α (EPAS) is found primarily in the endothelium, similar to endothelin-1 (ET-1) and fms-like tyrosine kinase-1 (Flt-1), the expression of which is controlled by HREs. Camenisch et al. [161] identified a unique sequence alteration in both ET-1 and Flt-1 HBS not found in other HIF-1 target genes, implying that these HBS might cause binding of HIF-2 rather than HIF-1. However, electrophoretic mobility shift assays showed HIF-1 and HIF-2 DNA complex formation with the unique ET-1 HBS about equal. Both DNA-binding and hypoxic activation of reporter genes using the ET-1 HBS were decreased compared with transferrin and EPO HBS. The Flt-1 HBS, in addition, was nonfunctional when assayed in isolation, suggesting that additional factors are required for hypoxic upregulation via the reported Flt-1 HRE [161]. Interestingly, HIF-1 activity could be restored fully by point mutat-

**Fig. 8.** The regulation of HIF by the prolyl hydroxylase enzyme, a putative oxygen sensor. VHL gene product (pVHL) interacts with HIF-1α and is required for the destruction of HIF-1α at the ODDD under normoxic conditions. HIF – pVHL interaction depends on both oxygen and iron availability. Furthermore, HIF-1α – pVHL interaction requires enzymatic posttranslational hydroxylation of HIF-1α at a single proline. This prolyl hydroxylation requires, besides oxygen and iron, also a citric acid cycle intermediate 2-oxoglutarate. Together with the HIF-induced activation of glucose and iron metabolism genes, it creates a tight link between oxygen sensing and cellular control of metabolism. Three novel human prolyl hydroxylases (PHDs) that modify HIF-1α have been characterized. Their activity is regulated by oxygen, 2-oxoglutarate, and iron availability, suggesting that PHDs may function as cellular oxygen sensors.
ing the ET-1 (but not the Flt-1) HBS, suggesting that the wild-type ET-1 HBS attenuate the full hypoxic response known from other oxygen-regulated genes [161–163].

4.1.2. HIF and MAPK regulation

Adaptive responses to hypoxia involve the regulation of gene expression by HIF, whose expression, stability, and transcriptional activity increase exponentially on lowering the partial pressure of oxygen (pO2) [5–8,163]. Tumor angiogenesis, the development of new blood vessels, is a highly regulated process that is controlled genetically by alterations in oncogene and tumor-suppressor gene expression and physiologically by the tumor microenvironment (hypoxia). Previous studies indicated that the angiogenic switch in Ras-transformed cells might be physiologically promoted by the tumor microenvironment through the induction of the angiogenic mitogen, VEGF.

HIF-1 controls the expression of a number of genes such as VEGF and EPO in low-oxygen conditions [164–167]. However, the molecular mechanisms that underlie the activation of the limiting subunit, HIF-1α, are still poorly resolved. In this respect, Mazure et al. [168] showed that Ras-transformed cells do not use the downstream effectors c-Raf-1 or MAPK in signaling VEGF induction by hypoxia, as overexpression of kinase-defective alleles of these genes did not inhibit VEGF induction under low-oxygen conditions. In contrast to the c-Raf-1/MAPK pathway, hypoxia increased phosphatidylinositol 3-kinase activity in a Ras-dependent manner, and the inhibition of phosphatidylinositol 3-kinase 3-kinase activity, genetically and pharmacologically, resulted in the inhibition of VEGF induction [168]. It was proposed that hypoxia modulates VEGF induction in Ras-transformed cells through the activation of a stress-inducible phosphatidylinositol 3-kinase/Akt pathway and the HIF-1 transcriptional response element.

On the mechanism of MAPK-dependent regulation of VEGF, it was demonstrated that the MAPK\textsuperscript{ERK1/2} signaling cascade controls VEGF expression at two levels. In normoxic conditions, MAPKs activate the VEGF promoter at the proximal (−88/−66) region where substance P (Sp)-1/activating protein (AP)-2 factors bind [83]. Activation of MAPK\textsuperscript{ERK1/2} was sufficient to turn on VEGF mRNA. Furthermore, MAPK\textsuperscript{ERK1/2} induced the phosphorylation of HIF-1α in vitro and HIF-1-dependent VEGF gene expression was strongly enhanced by the exclusive activation of MAPK\textsuperscript{ERK1/2}. Of note, the regulation of MAPK\textsuperscript{ERK1/2} activity was critical for controlling the proliferation and growth arrest of vascular endothelial cells at confluency [169]. Taken together, these data pointed to major targets of angiogenesis where MAPK\textsuperscript{ERK1/2} might exert a determinant action [170,171].

Furthermore, preliminary results showing that endogenous HIF-1α migrated 12 kDa higher than in vitro-translated protein led Richard et al. [172] to evaluate the possible role of phosphorylation on this phenomenon. In this respect, it was reported that HIF-1α was strongly phosphorylated in vivo and that its phosphorylation was responsible for the marked differences in the migration pattern of HIF-1α. In vitro, HIF-1α was phosphorylated by MAPK\textsuperscript{ERK1/2} and not by MAPK\textsuperscript{p38} or MAPK\textsuperscript{JNK} [172]. Interestingly, MAPK\textsuperscript{ERK1/2} stoichiometrically phosphorylated HIF-1 in vitro, as judged by a complete upper shift of HIF-1α. More importantly, it was demonstrated that the activation of the MAPK\textsuperscript{ERK1/2} pathway in quiescent cells induced the phosphorylation and shift of HIF-1α, which was abrogated in the presence of the MEK inhibitor, PD-98059.

In addition, with a VEGF promoter mutated at sites shown to be MAPK-sensitive (SP-1/AP-2-88-66 site), MAPK\textsuperscript{ERK1/2} activation was sufficient to promote the transcriptional activity of HIF-1 [172]. This interaction between HIF-1α and MAPK\textsuperscript{ERK1/2} indicated a cooperation between hypoxic and growth factor signals that ultimately leads to the increase in HIF-1-mediated gene expression. In support of the aforementioned observations, it was also demonstrated that in human microvascular endothelial cells-1 (HMEC-1), ERK kinases were activated during hypoxia. Using dominant negative mutants, Minet et al. [173, 174] concluded that MAPK\textsuperscript{ERK1} was needed for HIF-1 transactivation activity. Moreover, the same group further showed that HIF-1α was phosphorylated in hypoxia by a MAPK\textsuperscript{ERK1}-dependent pathway, suggesting a role for MAPK signaling in the transcriptional response to hypoxia.

The elucidation of the molecular mechanisms governing the transition from a nonangiogenic to an angiogenic phenotype is central for understanding and controlling malignancies. Viral oncogenes repre-
sent powerful tools for disclosing transforming mechanisms, and they may also afford the possibility of investigating the relationship between transforming pathways and angiogenesis. In this regard, Sodhi et al. [175,176] have recently observed that a constitutively active G-protein-coupled receptor (GPCR) encoded by the Kaposi’s sarcoma-associated herpes virus (KSHV)/human herpes virus (HHV)-8 was oncogenic and further stimulated angiogenesis by increasing the secretion of VEGF, which is a key angiogenic stimulator and a critical mitogen for the development of Kaposi’s sarcoma. KSHV GPCR enhanced the expression of VEGF by stimulating the activity of HIF-1α, which, in turn, activated transcription from an HRE within the 5′-flanking region of the VEGF promoter. Interestingly, stimulation of HIF-1α by the KSHV GPCR involved the phosphorylation of its regulatory/inhibitory domain by the MAPKp38 signaling pathway, thereby enhancing its transcriptional activity. Moreover, specific inhibitors of the MAPKp38 (SKF-86002) and MAPK<sup>ERK1/2</sup> (PD-98059) pathways were able to inhibit the activation of the transactivating action of HIF-1α induced by the KSHV GPCR, as well as the VEGF expression and secretion in cells over-expressing this receptor, suggesting that the KSHV GPCR oncogene subverts convergent physiological pathways leading to angiogenesis and provides insight into a mechanism whereby growth factors and oncogenes acting upstream from MAPK, as well as inflammatory cytokines and cellular stresses that activate MAPKp38, can interact with the hypoxia-dependent machinery of angiogenesis [175,176].

Oncogenic transformation and hypoxia induced glut-1 mRNA (glucose transporter during hypoxia). In this regard, the interaction between the Ras oncogene and hypoxia in upregulating glut-1 mRNA levels using Rat1 fibroblasts transformed with H-Ras (Rat1–Ras) was investigated. Transformation with H-Ras led to a substantial increase in glut-1 mRNA levels under normoxic conditions and additively increased glut-1 mRNA levels in concert with hypoxia [177]. Using a luciferase reporter construct containing 6 kb of the glut-1 promoter, it was shown that this effect was mediated at the transcriptional level. Promoter activity was much higher in Rat1–Ras cells than in Rat1 cells, and could be downregulated by cotransfection with a dominant negative Ras construct (Ras-N17). In addition, a 480-bp cobalt/hypoxia-responsive fragment of the promoter containing a HIF-1 binding site showed significantly higher activity in Rat1–Ras cells than in Rat1 cells, suggesting that Ras might mediate its effect through HIF-1 even under normoxic conditions.

Consistent with this, Rat1–Ras cells displayed higher levels of HIF-1α protein under normoxic conditions. In addition, a promoter construct containing a 4-bp mutation in the HIF-1 binding site showed lower activity in Rat1–Ras cells than a construct with an intact HIF-1 binding site. The activity of the latter construct, but not the former, could be downregulated by Ras-N17, supporting the significance of the HIF-1 binding site in regulation by Ras [177]. Of note, the PI3K inhibitor LY29004 downregulated glut-1 promoter activity and mRNA levels under normoxia and also decreased HIF-1α protein levels. Furthermore, the MAPK<sup>ERK1/2</sup> cascade, known to phosphorylate HIF-1α, did not modulate the degradation/stabilization profile of HIF-1α. However, recent evidence suggested that the rate of HIF-1α degradation depends on the duration of hypoxic stress. In this respect, the degradation of HIF-1α was suppressed by: (i) inhibiting general transcription with actinomycin D, or (ii) specifically blocking HIF-1-dependent transcriptional activity [178].

In keeping with these findings, it was postulated that HIF-1α might be targeted to the proteasome via a HIF-1α proteasome-targeting factor (HPTF), whose expression was directly under the control of HIF-1-mediated transcriptional activity. Although HPTF is not yet molecularly identified, it is clearly distinct from the pVHL. In preference to these observations, recent results also indicated that the stabilization of HIF-1α protein, by treatment of proteasome inhibitors, was not sufficient for hypoxia-induced gene activation, and an additional hypoxia-dependent modification was found necessary for gene expression by HIF-1α [179]. In this respect, it was demonstrated that PD-98059 did not change either the stabilization or the DNA-binding ability of HIF-1α, but it inhibited the transactivation ability of HIF-1α, thereby reducing the hypoxia-induced transcription of both an endogenous target gene and a hypoxia-responsive reporter gene. Furthermore, hypoxia induced MAPK<sup>ERK1/2</sup> phosphorylation and the expression of dominant-negative MAPK<sup>ERK1/2</sup> mutants reduced HIF-1-dependent transcription of the hypoxia-responsive reporter gene, suggesting that the hypoxia-induced transactivation
ability of HIF-1α may be regulated by different mechanisms than its stabilization and DNA-binding, and that these processes can be, at least in part, experimentally dissociated [179]. These observations led to the conclusion that MAPK<sub>ERK1/2</sub> could regulate the transactivation, but not the stabilization or DNA-binding ability, of HIF-1α.

In support of this role of MAPK in HIF-1 stabilization and DNA-binding activity, Tacchini et al. [180] recently reported that hepatocyte growth factor (HGF), a multifunctional cytokine of mesenchymal origin, has the potential to activate the DNA binding of HIF-1α in the HepG2 cell line. An increased expression of HIF-1α (mRNA and nuclear protein levels) was also observed. To investigate the molecular basis of the HIF-1 response under this non-hypoxic condition, the expression of putative target genes was evaluated. It was found that a time-dependent increase in steady-state mRNA levels of heme oxygenase and urokinase plasminogen activator followed by that of urokinase receptor was evident. The enhanced expression of these genes might confer the invasive phenotype, since HGF is a proliferative and scatter factor [180]. In addition, HIF-1 activity and its regulation in HGF-treated cells were followed: (i) the activation of HIF-1 DNA binding was prevented by proteasome blockade, probably because stabilization of the cytosolic α-subunit protein level was not sufficient to generate a functional form (also under these conditions, nuclear protein level of HIF-1α did not increase); (ii) N-acetyl-L-cysteine (NAC), a free radical scavenger and a GSH precursor, strongly decreased HIF-1 activation, suggesting a role of ROS in this process; and (iii) the thiol-reducing agent dithiothreitol (DTT) was ineffective. Consistent with these observations, NAC reduced the stimulatory effect of HGF on stress kinase activities, while MAPK<sub>ERK1/2</sub> was unmodified, suggesting an involvement of MAPK<sub>JNK</sub> and MAPK<sub>P38</sub> in HIF-1 activation [180]. Of interest, LY294002 caused the blockade of PI3K and prevented the enhancement of HIF-1 DNA binding and MAPK<sub>JNK</sub> activity; the inhibition of MAPK<sub>ERK1/2</sub> phosphorylation was rather ineffective.

Environmental signals in the cellular milieu such as hypoxia, growth factors, extracellular matrix (ECM), or cell surface molecules on adjacent cells can activate signaling pathways that communicate the state of the environment to the nucleus. Several groups have evaluated gene expression or signaling pathways in response to increasing cell density as an in vitro surrogate for in vivo cell–cell interactions. These studies have also perhaps assumed that cells grown at various densities in standard in vitro incubator conditions do not have different pericellular oxygen levels [180,181]. However, pericellular hypoxia can be induced by increasing cell density, which can exert profound influences on the target cell lines and may explain a number of findings previously attributed to normoxic cell–cell interactions. Thus, the hypothesis that cell–cell interactions, as evaluated by the surrogate approach of increasing in vitro cell density in routine normoxic culture conditions, results in pericellular hypoxia in prostate cancer cells was examined. A recent report indicated that paracrine cell interactions could induce nuclear localization of HIF-1α protein and that this translocation was associated with strong stimulation of the HRE reporter activity [181]. Moreover, cell density-induced activity of the HRE was dependent on NO production, which acts as a diffusible paracrine factor secreted by densely cultured cells, suggesting that cell interactions associated with pericellular hypoxia might lead to the physiological induction of HRE activity via the cooperative action of Ras, MEK1, and HIF-1α via pericellular diffusion of NO. The role of hypoxia and MAPK signaling pathways in the regulation of HIF-1 is schematized in Fig. 9.

4.2. MAPK-mediated regulation of nuclear factor-κB (NF-κB)

Although the transcription factor NF-κB has been originally recognized in regulating gene expression in B-cell lymphocytes [182], subsequent investigations have demonstrated that it is one member of a ubiquitously expressed family of Rel-related transcription factors that serve as critical regulators of many genes, including those of proinflammatory cytokines [183–201]. NF-κB comprises the Rel family of inducible transcription factors that are key mediators in regulating the progression of the inflammatory process [202–213]. Therefore, the activation and regulation of the NF-κB/Rel transcription family, via nuclear translocation of cytoplasmic entities and complexes, play a central role in the evolution of inflammation through the regulation of genes essentially involved in encoding...
proinflammatory cytokines and other inflammatory mediators [214–227].

The NF-κB/Rel family includes five members: NF-κB1 (p50/p105 {p50 precursor}), NF-κB2 (p52/p100 {p52 precursor}), RelA (p65), RelB (p68), and c-Rel (p75). Despite the ability of most Rel members (with the exception of p68) to homodimerize, as well as to form heterodimers, with each other, the most prevalent activated form of NF-κB is the heterodimer p50-p65, which possesses the transactivity domains necessary for gene regulation. The NF-κB members contain a Rel homology domain (RHD), which is responsible for dimer formation, nuclear translocation, sequence-specific consensus DNA recognition, and interaction with inhibitory κB (IκB) proteins, which are the cytosolic inhibitors of NF-κB.

The translocation and activation of NF-κB in response to various stimuli are sequentially organized at the molecular level. In resting, unstimulated cells, NF-κB resides in the cytoplasm as an inactive NF-κB/IκB complex, a mechanism that hinders the recognition of the nuclear localization signal (NLS) by the nuclear import machinery, thereby retaining the NF-κB complex within the cytosol. In its inactive state, the heterodimeric NF-κB, which is mainly composed of two subunits, p50 (NF-κB1) and p65 (RelA), is present in the cytoplasm associated with IκB [225–228]. Upon stimulation, such as with cytokines and lipopolysaccharide endotoxin (LPS), derived from the cell wall of Gram-negative bacteria, IκB-α, the major cytosolic inhibitor of NF-κB, undergoes phosphorylation on serine/threonine residues, ubiquitination, and

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Fig. 9. The role of hypoxia and MAPK signaling pathways in the regulation of HIF-1 and HIF-1-dependent gene transcription (see text for further discussion).
subsequent proteolytic degradation, thereby unmasking the NLS on p65 and allowing nuclear translocation of the complex. This sequential propagation of signaling ultimately results in the release of NF-κB subunits from the IκB-α inhibitor, allowing translocation and promotion of gene transcription.

Signals emanating from membrane receptors, such as those for IL-1 and TNF-α, activate members of the MEKK-related family, including NF-κB-inducing kinase (NIK) and MEKK-1, both of which are involved in the activation of IκB kinases, IKK1 and IKK2, components of the IKK signalosome. These kinases phosphorylate members of the IκB family, including IκB-α, the major cytosolic inhibitor of NF-κB, at specific serines within their amino termini, thereby leading to site-specific ubiquitination and degradation by the proteasome. This sequential trajectory culminating in the inducible degradation of IκB, which occurs through consecutive steps of phosphorylation and ubiquitination, allows freeing of the NF-κB complex, which translocates onto the nucleus, binds specific κB moieties, and initiates gene transcription (Fig. 10).

Hypoxia can cause the activation of NF-κB and the phosphorylation of its inhibitory subunit, IκB-α. For instance, Beraud et al. [228] reported that hypoxia, reoxygenation, and the tyrosine phosphatase inhibitor pervanadate activate NF-κB via a mechanism involving the phosphorylation of IκB-α on tyrosine residues. This modification, however, did not lead to degradation of IκB-α by the proteasome/ubiquitin pathway, as evident from the stimulation of cells with proinflammatory cytokines. It was shown that p85-α, the regulatory subunit of phosphatidylinositol 3-kinase, specifically associates through its Src homology 2 domains with tyrosine-phosphorylated IκB-α in vitro and in vivo after stimulation of T cells with pervanadate [227]. This association could provide a mechanism by which newly tyrosine-phosphorylated IκB-α is sequestered from NF-κB.

Another mechanism by which phosphatidylinositol 3-kinase contributed to NF-κB activation in response to pervanadate appeared to involve its catalytic p110 subunit. This was evident from the inhibition of pervanadate-induced NF-κB activation and reporter gene induction by treatment of cells with nanomolar amounts of a phosphatidylinositol 3-kinase inhibitor, Wortmannin. The compound had virtually no effect on TNF- and IL-1-induced NF-κB activities. In addition, Wortmannin did not inhibit the tyrosine phosphorylation of IκB-α or alter the stability of the phosphatidylinositol 3-kinase complex, but inhibited Akt kinase activation in response to pervanadate, suggesting that both the regulatory and the catalytic subunit of phosphatidylinositol 3-kinase play a role in NF-κB activation by the tyrosine phosphorylation-dependent pathway [228].

These observations were also supported by Canty et al. [229] who reported assays that also showed substantial NF-κB activation in hypoxic HUVECs after reoxygenation and in cultures treated with H₂O₂. Pervanadate also induced marked NF-κB activation in HUVECs, indicating that H₂O₂-induced NF-κB activation is potentiated by the inhibition of tyrosine phosphatases. Western blotting of cytoplasmic IκB-α demonstrated that NF-κB activation induced by oxidative stress was not associated with IκB-α degradation. In contrast, however, TNF-α-induced NF-κB activation occurred in concert with IκB-α degradation. The compound had virtually no effect on

Fig. 10. NF-κB signal transduction pathway. Various incoming signals converge on the activation of the IκB kinase (IKK) complex. IKK then phosphorylates IκB at two N-terminal serines, which signals it for ubiquitination and proteolysis (proteasome). Freed NF-κB complex (p50/p65) enters the nucleus and activates gene transcription.
degradation. Furthermore, inhibition of IκB-α degradation with a proteasome inhibitor, MG-115, blocked NF-κB activation induced by TNF-α; however, MG-115 had no effect on NF-κB activation during oxidative stress.

With the use of dominant negative mutants of Ha-Ras and Raf-1, Koong et al. [230] investigated some of the early signaling events leading to the activation of NF-κB by hypoxia. Both dominant negative alleles of Ha-Ras and Raf-1 inhibited NF-κB induction by hypoxia, suggesting that the hypoxia-induced pathway of NF-κB induction is dependent on Ras and Raf-1 kinase activity. Furthermore, although conditions of low oxygen can also activate MAPK_{ERK1/2}, these kinases did not appear to be involved in regulating NF-κB by hypoxia, an observation supported by the notion that the dominant negative mutants of MAPK had no inhibitory effect on NF-κB activation by hypoxia [230]. Moreover, an increase in Src proto-oncogene activity of cellular exposure to hypoxia was observed under similar conditions. It was subsequently postulated that Src activation by hypoxia might be one of the earliest events that precedes Ras activation in the signaling cascade that ultimately leads to the phosphorylation and dissociation of the inhibitory subunit of NF-κB, IκB-α.

Similarly, the inhibitor of MAPK_{ERK1/2} (PD-98059) blocked CoCl₂-induced NF-κB activation and VCAM-1 expression (CoCl₂ acts as a mimic molecule for hypoxia to study cellular signaling pathways) [231]. These aforementioned mechanisms were reinforced with observations related to a stress-induced cytokine, termed cardiotrophin-1 (CT-1), which belongs to the IL-6/glycoprotein 130 receptor-coupled cytokine family. CT-1 is released from the heart in response to hypoxic stress and it protects cardiac myocytes from hypoxia-induced apoptosis, thus establishing a central role for this cytokine in the cardiac stress response [232]. It was observed that, in cardiac myocytes, CT-1 activated MAPK_{p38} and MAPK_{ERK} as well as Akt. CT-1 also induced the degradation of the NF-κB cytosolic anchor, IκB-α, as well as the translocation of the RelA (p65) subunit (the major transactivating member of the Rel family) of NF-κB to the nucleus and increased expression of an NF-κB-dependent reporter gene. Furthermore, inhibitors of the MAPK_{p38}, MAPK_{ERK}, or Akt pathways each partially reduced CT-1-mediated NF-κB activation, as well as the cytoprotective effects of CT-1 against hypoxic stress. Together, the inhibitors completely blocked CT-1-dependent NF-κB activation and cytoprotection, indicating that CT-1 signals through MAPKs and Akt in a parallel manner to activate NF-κB. The role of hypoxia and MAPK signaling pathways in the regulation of NF-κB and NF-κB-dependent gene transcription is schematized in Fig. 11.

![Fig. 11. The role of hypoxia and MAPK signaling pathways in the regulation of NF-κB and NF-κB-dependent gene transcription.](image-url)
4.3. MAPK-mediated regulation of activating protein-1 (AP-1)

Pathophysiological hypoxia is an important modulator of gene expression in solid tumors and other pathologic conditions. In this respect, it was observed that transcriptional activation of the c-jun proto-oncogene in hypoxic tumor cells correlates with the phosphorylation of the ATF-2 transcription factor [233]. This finding suggested that hypoxic signals transmitted to c-jun involve protein kinases that target AP-1 complexes (c-Jun and ATF-2) that bind to its promoter region. Stress-inducible protein kinases capable of activating c-jun expression include MAPKJNK and MAPKp38. Transient activation of SAPK/JNKs occurred by tumor-like hypoxia concurrent with the transcriptional activation of MKP-1, a stress-inducible member of the MAPK phosphatase (MKP) family of dual-specificity protein tyrosine phosphatases. Of interest is the observation that Northern blots showed an increase in the level of c-jun and c-fos subunits during hypoxia. Gel mobility shift analysis of nuclear extracts from hypoxia-exposed cells showed an increase in AP-1 binding activity [234]. In addition, hypoxic treatment strongly activated MAPKJNK-1, thereby leading to phosphorylation and activation of c-Jun. Furthermore, expression of a dominant negative mutant of MAPKJNK-1 suppressed hypoxia-induced MAPKJNK-1 activation as well as reporter gene expression.

Unequivocally, using antisense c-fos strategy, it was shown that c-fos is essential for the activation of AP-1 complex and subsequent stimulation of downstream genes [235]. Furthermore, hypoxia caused Ca\(^{2+}\) influx through L-type voltage-gated Ca\(^{2+}\) channels and the hypoxia-induced c-fos gene expression was Ca\(^{2+}\)/calmodulin-dependent. It was also demonstrated that hypoxia induced the activation of MAPKERK and MAPKp38, but not MAPKJNK, contrary to observations of Le and Corry [234]. The phosphorylation of MAPKERK was found to be essential for c-fos activation via serum response element (SRE) cis-element. Further characterization of nuclear signaling pathways provided evidence for the involvement of Src, a nonreceptor PTK, and Ras, a small G-protein, in the hypoxia-induced c-fos gene expression, suggesting a possible role for nonreceptor PTKs in propagating signals from G-protein-coupled receptors to the activation of immediately-early genes such as c-fos during hypoxia.

In cancerous HeLa cells, hypoxic conditions have been reported to induce the transcriptional activation of c-fos transcription via the SRE [236]. Mutations in the binding site for the ternary complex factor Elk-1 and SRE abolished this induction, indicating that a ternary complex at the SRE is necessary for the induction of the c-fos gene under hypoxia. The transcription factor Elk-1 was covalently modified by phosphorylation in response to hypoxia. Furthermore, this hyperphosphorylation of Elk-1, the activation of MAPK, and the induction of c-fos transcripts were blocked by PD-98059. An in vitro kinase assay with Elk-1 as substrate showed that MAPK is activated under hypoxia. Furthermore, the activation of MAPK corresponded temporally with the phosphorylation and activation of Elk-1 [236]. On mechanisms, a decrease of intracellular ROS level by hypoxia induced c-fos via the MAPK pathway, suggesting that the intracellular redox levels may be directly coupled to tumor growth, invasion, and metastasis via Elk-1-dependent induction of c-fos-controlled genes [236,237]. The role of hypoxia and MAPK signaling pathways in the regulation of AP-1 is shown in Fig. 12.

![Fig. 12. The role of hypoxia and MAPK signaling pathways in the regulation of AP-1 and AP-1-dependent gene transcription via the cytokine-dependent pathway.](image-url)
5. The role of MAPK signaling pathways in hypoxia- or anoxia-tolerant organisms

Freshwater turtles, such as the western-painted turtles *Chrysemys picta bellii* and the hatchling red-eared turtles *Trachemys scripta elegans*, are considered among the most anoxia-tolerant, air-breathing vertebrates [238–243]. Integrative and sustained adaptations on the imposition of submergence anoxia underlie the animal’s capacity to tolerate these conditions for long periods of time [244–246]. *C. picta bellii* and *T. scripta elegans* are unusually tolerant of anoxia in that they survive 24–48 h of anoxia at 25 °C and 4–5 months at 2–3 °C during winter dormancy. Survival of neurons in these remarkable turtles involves a profound reduction in energy me-

![Fig. 13. Hypoxia-mediated regulation of MAPK<sup>p38</sup> signaling pathway. (A) Exposure to hypoxia for a period of time ranging from 5 h to 6 weeks did not significantly affect the phosphorylation/activation of MAPK<sup>p38</sup>, as compared with normoxia control. (B) Hypoxia/reoxygenation for 6 h variably affected the phosphorylation/activation of MAPK<sup>p38</sup>, with suppression at 1 day and 1 week of hypoxia followed by oxygenation. This MAPK<sup>p38</sup> suppression was not observed at either 3 days or 6 weeks of hypoxia. The lower panel shows the expression of the nonphosphorylated form of MAPK<sup>p38</sup>, as verification for semiquantitative loading in parallel lanes. (C) Histogram analysis of the relative abundance of the phosphorylated form of MAPK<sup>p38</sup> under hypoxia/reoxygenation (6 h). (The level of expression at normoxia was adjusted to one unit and all other values were expressed relative to this unit.) ND, not determined; *n* = 2–5, which designates the number of turtles and independent experiments performed for each variable.]
metabolism to approximately 10–20% of the normoxic rate at the same temperature [245–247], suggesting a coordinated reduction of ATP-generating mechanisms and ATP-consuming pathways. This metabolic ‘arrest’ has been shown to lead to suppression of ion channels, thereby allowing decreased excitability, reduced ion translocation, and preservation of [ATP] during the energetic stress imposed by anaerobic conditions [248]. Furthermore, other targets have been suppressed, including numerous enzymes and molecules that regulate protein synthesis [249,250]. Another feature that characterizes survival is the ability to

![Diagram](image)

**Fig. 14.** Hypoxia-mediated regulation of MAPK<sup>ERK1/2</sup> (MAPK<sup>p44/p42</sup>) signaling pathway. (A) Exposure to hypoxia for a period of time ranging from 5 h to 6 weeks variably and in a biphasic manner affected the phosphorylation/activation of MAPK<sup>p44/p42</sup>, as compared with normoxia control. Whereas 5 h of hypoxia significantly increased the phosphorylation/activation of MAPK<sup>p44/p42</sup>, 1 day to 1 week was not significantly different from normoxia, and at 6 weeks of hypoxia, the phosphorylation/activation of MAPK<sup>p44/p42</sup> was suppressed, as compared with normoxia and 5 h. (B) Hypoxia/reoxygenation for 6 h mildly affected the phosphorylation/activation of MAPK<sup>p44/p42</sup>, with suppression at 6 weeks of hypoxia followed by oxygenation. This MAPK<sup>p44/p42</sup> suppression was not observed at 1 day to 1 week of hypoxia. The lower panel shows the expression of the nonphosphorylated form of MAPK<sup>p44/p42</sup>, as verification for semiquantitative loading in parallel lanes. (C) Histogram analysis of the relative abundance of the phosphorylated form of MAPK<sup>p44/p42</sup> under hypoxia/reoxygenation (6 h). (The level of expression at normoxia was adjusted to one unit and all other values were expressed relative to this unit.) ND, not determined; n = 2–5, which designates the number of turtles and independent experiments performed for each variable.
buffer an acid–base equilibrium in response to lactate accumulation due to anaerobic glycolysis [250]. This latter mechanism is centered on the release of carbonates from the bone and shell to enhance body fluid buffering of lactic acid. Therefore, the combination of slow metabolic activity and responsive mineral reserve is crucial to the survival of those animals under unprecedented conditions.

The biochemical and physiological mechanisms of anoxia tolerance in turtles have been previously examined at the level of ion transport and ATP turnover to better understand the effect of oxygen deprivation [251]. However, changes in the phosphorylation state of key enzymes and kinases may occur during anoxia; therefore, reversible protein phosphorylation could be a critical factor and major mechanism of metabolic reorganization for enduring anaerobiosis [251,252]. For instance, it has been shown that anoxia mediated changes in the activities of PKA, PKC, and protein Ppase-1 [253]. Furthermore, anoxia was shown to impose variations in protein synthesis, mRNA accumulation, and gene transcription in turtle organs, suggesting that upregulation of selective genes is crucial for surviving anoxia.

The role of two vertebrate MAPKs in mediating responses to in vivo anoxia or freezing exposures was examined in four organs (liver, heart, kidney, and brain) of hatchling red-eared turtles, *T. scripta elegans*, which are naturally tolerant of these stresses [250,251]. The extracellular signal-regulated kinases (MAPK ERK1/2) were not stress-activated except in the brain of frozen turtles. MAPK JNK was transiently activated by anoxia exposure in all four organs (after 1 h in brain or 5 h in other organs), but activity was suppressed during freezing except in the brain, which showed a transient activation of MAPK JNK after 1 h. In addition, changes in the concentrations of the transcription factors, c-fos and c-myc, were also stress- and organ-specific. These results for an anoxia-tolerant animal suggested the potential importance of the MAPKs and immediate-early genes (c-fos, c-myc) in mediating adaptive responses to oxygen deprivation.

![Fig. 15. Hypoxia-mediated regulation of antiapoptotic cofactor, Bcl-2, and proapoptotic cofactor, Bax. (A) Exposure to hypoxia for a period of time ranging from 5 h to 6 weeks mildly upregulated the protein expression of Bcl-2 at 1 day to 1 week of hypoxia, as compared with normoxia control. (B) Hypoxia/reoxygenation for 6 h has no effect on Bcl-2 expression. (C) Exposure to hypoxia for a period of time ranging from 5 h to 6 weeks upregulated the protein expression of Bax at 3 days and 1 week of hypoxia, as compared with normoxia control. (D) Hypoxia/reoxygenation for 6 h variably affected Bax expression, with upregulation at 1 day, 3 days, and 1 week of hypoxia followed by oxygenation.](image-url)
Recently, Haddad et al. have investigated in vivo the hypoxia-mediated regulation of MAPK signaling pathways and caspase/apoptosis cofactor expression in anoxia-tolerant turtles, *C. picta bellii* (unpublished observations). Whereas hypoxia has no apparent effect on MAPKp38 phosphorylation, hypoxia–reoxygenation suppressed this pathway in early hypoxia (Fig. 13). In contrast, hypoxia-mediated phosphorylation of MAPKERK followed a biphasic module in that there was enhancement at early hypoxia followed by suppression at late hypoxia, similar to the effect observed with MAPKJNK (Fig. 14). Of interest, neither hypoxia nor hypoxia–reoxygenation mediated the activation of the caspase pathway; however, hypoxia upregulated the expression of Bax and, to a lesser extent, Bcl-2, an effect potentiated with reoxygenation, indicating the involvement of a Bax-sensitive pathway (Fig. 15) (Haddad et al., unpublished observations, UCSF). In summary, these results indicate that the regulation of MAPK signaling pathways in anoxia-tolerant turtles is inharmonious, and that apoptosis regulation is caspase-insensitive and requires, at least in part, the involvement of a Bax-dependent mechanism. The patterns of MAPK activation in a stress-tolerant animal suggest the relative importance of these kinase pathways in cellular adaptation to oxygen deprivation or freezing and identify novel natural activators of MAPKs in vivo. The specificity of the signaling pathways is also emphasized here as general whole body stresses (anoxia and freezing) activated individual MAPKs in a tissue-, time-, and stress-dependent manner. Hypoxia-mediated regulation of MAPK signaling in anoxia-tolerant turtles is shown in Fig. 16.

6. Summary, conclusion, and future prospects

Hypoxia is a crucial signal in development and physiology, but it may well be a key element in the development of many abnormalities and disease conditions [254,255]. The complex signaling pathways mediated by hypoxia revolve around the hinge of controlling gene transcriptions by upstream effectors and regulators. This feedforward mode of gene regulation involves the revolving axis of MAPK signaling molecules, which coordinate messages from cell receptors to effector cofactors situated at different levels within the intriguing hierarchy of cell regulation. Not only are MAPKs regulated by hypoxia signaling, but also they play a crucial role in mediating hypoxia-dependent gene regulation, hence the evolution of a revolving axis for cell regulation [256–259]. In so far as the underlying mechanisms controlling this multifaceted revolving axis of gene regulation are vaguely comprehended, the basis for complex interactions between MAPK modules and the various components of the cell machinery has already paved the way to developing a strategy for understanding not only the processes of development but also the evolution of pathophysiologic conditions stemming out of the malfunctioning of these systems. The burgeoning, yet central, role of hypoxia MAPK modules as a major determinant of many life-critical cell functions may perhaps allow us to understand the complex mechanisms in display mediating the regulation of transcription and gene expression for better therapeutic pharmacologic interventions [260–272].

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