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Nitric oxide, oxidative stress, and apoptosis

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Nitric oxide, oxidative stress, and apoptosis. Life demands intra- and intercellular communication in and between cells to respond and adapt to changes in the environment. Among signaling molecules, reactive oxygen (ROS) and nitrogen (RNS) species gained attention in facilitating intracellular communication and causing cell demise during pathology. Complexity was added with the notion that ROS and RNS signals overlap and/or produce synergistic, as well as antagonistic, effects. This is exemplified by using oxidized lipoproteins (oxLDL), or NO donors, in provoking the stabilization of two well recognized transcription factors, such as tumor suppressor p53 and hypoxia-inducible factor-1α (HIF-1α). Radical (i.e., superoxide) (O_2^-) formation in response to oxLDL is associated with p53, as well as HIF-1α accumulation in human macrophages, a process that is antagonized by NO. On the other side, NO-elicited HIF-1α stabilization is modulated by O_2^-. Thus, ROS- and RNS-signaling is important in understanding cell physiology and pathology, with the notion that marginal changes in the flux rates of either NO or O_2^- may shift vital signals used for communication into areas of pathology in close association with human diseases.

OXLDL AND PPARγ IN SUPEROXIDE FORMATION AND SIGNALING

Low-density lipoproteins acquire a number of important pathophysiologic activities as a result of oxidative modification [1]. Oxidation is achieved in vitro by autooxidation in the presence of transition metals or in vivo via cell-mediated mechanisms. Oxidized low-density lipoproteins (oxLDL) are powerful regulators of cell signaling in provoking various responses [2]. Among other activities, oxLDL is both a potent chemoattractant for circulating monocytes and a differentiating agent that promotes transition of macrophages to lipid-loaden foam cells. In close association, receptor-mediated endocytosis of oxLDL by several scavenger receptor family members, including macrophage class A scavenger receptor (SR-A), CD36, scavenger receptor that binds phosphatidylserine and oxidized lipoprotein (SR-PSOX), and lectin-like oxidized low-density lipoprotein receptor-1 (LOX1), is implicated in the process of atherogenesis. Atherosclerosis is now considered a problem of wound healing and chronic inflammation and can be viewed as a "response to injury" with lipoproteins, or other risk factors as the injurious agents with the important notion that accumulation of lipid-laden foam cells in fatty streaks are a primary event in disease progression. Increased oxidative stress participates in vascular dysfunction and atherogenesis [2]. Furthermore, oxLDL and/or ROS regulate pathways linked to apoptosis and affect gene expression by modulating a large number of transcription factors, one of which is the peroxisome proliferator-activated receptor gamma (PPARγ) [3].

Peroxisome proliferator-activated receptors (PPARs) are a group of lipid-activated nuclear receptors that heterodimerize with the 9-cis retinoic acid receptor (RXR) to form functional transcription factors that regulate genes involved in lipid and glucose metabolism. Examples include adipocyte fatty acid binding protein aP2, phosphoenolpyruvate carboxykinase, lipoprotein lipase, and the brown fat uncoupling protein UCP1. Activation of PPARγ is achieved by naturally occurring ligands that comprise derivatives of linoleic or arachidonic acid, or synthetic antidiabetic drugs known as thiazolidinediones.

We and others have demonstrated that membrane-bound NAD(P)H oxidase is a major source of ROS formation, mediated by oxLDL in macrophages [4]. The oxidative burst was measured by flow cytometry and quantitated by oxidation of the redox sensitive dye, dichlorodihydrofluorescein diacetate (DCF). Short-time stimulation dose-dependently elicited ROS formation. Diphenylene iodonium prevented ROS formation, thus pointing to the involvement of an NAD(P)H oxidase in producing reduced oxygen species. In contrast, preincubation of macrophages with oxLDL for 16 hours showed an attenuated oxidative burst after a second contact with oxLDL. Taking into account that oxLDL is an established PPARγ agonist, and considering the anti-inflammatory properties of PPARγ, we went on and showed that a PPARγ agonist such as ciglitazone attenuated ROS formation [5]. Along that line, major lipid peroxidation products of oxLDL, such as 9-HODE and 13-

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HODE, shared that performance. Supporting evidence that PPARγ activation accounted for reduced ROS generation came from studies in which PPRE decoy oligonucleotides, but not mutated oligonucleotides, supplied in front of oxLDL delivery were allowed to regain a complete oxidative burst upon cell activation [6]. We conclude that oxLDL not only elicits an oxidative burst upon first contact, but also promotes desensitization of macrophages via activation of PPARγ.

In some analogy, we noticed the ability of oxLDL to provoke p53 accumulation in human macrophages (primary monocyte derived macrophages and Mono Mac 6 cells) in close association with the appearance of apoptosis [7]. Apoptotic markers comprised staining of phosphatidylserine at the outer leaflet of the membrane (Annexin V staining), DNA fragmentation determined by the cell death detection ELISA, and histochemical features of chromatin condensation (i.e., DAPI-staining). Initiation of apoptosis, as well as p53 accumulation, was sensitive to diphenylene iodonium, thus correlating O_2^- formation upon oxLDL addition with downstream signaling events. We conclude that oxLDL, via ROS signaling, contributes to macrophage cell death (i.e., apoptosis while subtoxic concentrations of oxLDL alter the macrophage phenotype with the outcome of suppressed O_2^- formation). Thus, desensitization of macrophages by oxLDL may have important consequences for the behavior of macrophages and foam cells in atherosclerotic lesions.

HIF-1α ACCUMULATION BY REDOX SIGNALS

HIF-1 is a heterodimeric transcription factor composed of α and β subunits [8]. While HIF-1β is constitutively expressed in many cell types, HIF-1α is present under normal oxygen supply at undetectable amounts. Under these conditions, HIF-1α is rapidly degraded by the ubiquitin-proteasome system. Under hypoxic conditions, HIF-1α is stabilized. Following its heterodimerization with HIF-1β and translocation to the nucleus, binding to promoter-specific sites known as hypoxia response elements (HRE) drives classic hypoxia responsive genes. Recent studies suggest that alterations in the level of ROS provide a redox signal for HIF-1 induction by hypoxia [9]. Moreover, ROS also appear to regulate HIF-1 activity under normoxia. In some cell types, direct exposure to ROS or increased ROS production in response to hormones or growth factors has been shown to mediate HIF-1α protein accumulation and HIF-1-dependent transcription. Along that line, cytokine-mediated stabilization of HIF-1α and HIF-1 transactivation requires a ROS-sensitive pathway. We obtained evidence that oxLDL induced HIF-1α protein accumulation in human macrophages under normoxia. OxLDL-mediated HIF-1α accumulation was attenuated by pretreatment with the antioxidant N-acetyl-L-cysteine (NAC) and NAD(P)H oxidase inhibitors, such as diphenylene iodonium (DPI) or 4-(2-aminoethyl)-benzenesulfonyl fluoride (AEBSF), thus implicating the contribution of oxLDL-generated ROS. Furthermore, the most physiologic NO donor S-nitrosoglutathione (GSNO) inhibited oxLDL-induced HIF-1α accumulation. Based on HIF-1-dependent luciferase reporter gene analysis, we established oxLDL-elicited HIF-1 transactivation, which was again sensitive to DPI. Our results indicated that ROS are required for oxLDL to elicit a "hypoxic" response.

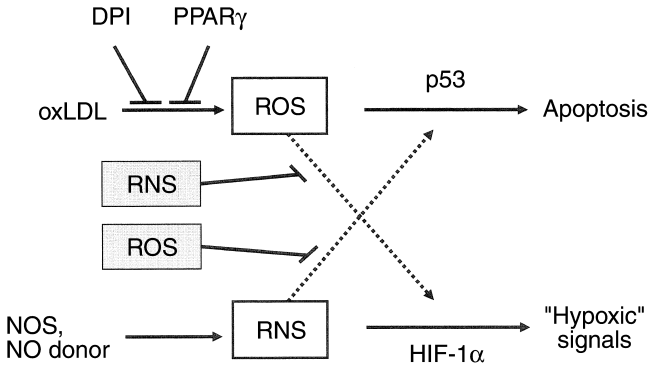

Fig. 1. HIF-1α and p53 stabilization under the influence of ROS/RNS. Stabilization of both HIF-1α and p53 under the influence of NO and O_2^- with indications of signal cross talk, sites of intervention, and potential pathophysiologic outcomes are shown (→ signal propagation; ⊣ inhibitory or suppressive). For details see the text.

Recently, nitric oxide emerged as a messenger with the ability to stabilize HIF-1α and to transactivate HIF-1 under normoxia [10, 11]. Considering that reactive nitrogen species are recognized for posttranslation protein modifications, among others S-nitrosation, we asked whether HIF-1α is a target for S-nitrosation. In vitro NO+ donating compounds such as GSNO and SNAP provoked massive S-nitrosation of purified HIF-1α. All 15 free thiol groups found in human HIF-1α are subjected to S-nitrosation. Thiol modification is not shared by spermine-NONOate, a NO radical donating agent. However, spermine-NONOate in the presence of O_2^-, generated by xanthine/xanthine oxidase, regained S-nitrosation, most likely via formation of a N_2O_3-like species. In RCC4 and HEK293 cells, GSNO or SNAP reproduced S-nitrosation of HIF-1α, with a significantly reduced potency, however. Importantly, endogenous formation of NO in RCC4 cells via inducible NO synthase elicited S-nitrosation of HIF-1α that was sensitive to inhibition of inducible NO synthase activity with NMMA. In conclusion, HIF-1α is a target for S-nitrosation by exogenously and endogenously produced NO in close association with NO-evoked protein stabilization.

CONCLUSION

NO and O_2^- are versatile messengers in physiology and pathology [12, 13] (Fig. 1). Signaling properties and

toxicity of both radicals are affected, to a large extent, by the existing biologic milieu, relative rates of NO/O_2^- formation, and scavenger systems [14]. For the future it will be essential to define the transition from "signaling" to "toxicity" and to use this knowledge for therapeutic interventions.

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REFERENCES

1. Glass CK, Witztum JL: Atherosclerosis: The road ahead. *Cell* 104:503–516, 2001
2. Cai H, Harrison DG: Endothelial dysfunction in cardiovascular diseases: The role of oxidant stress. *Circ Res* 87:840–844, 2000
3. Michalik L, Wahli W: Peroxisome proliferator-activated receptors: Three isotypes for a multitude of functions. *Curr Opin Biotechnol* 10:564–570, 1999
4. Fischer B, von Knethen A, Brüne B: Dualism of oxLDL in provoking and attenuating the oxidative burst in macrophages: Role of PPARγ. *J Immunol* 168:2828–2834, 2002
5. von Knethen A, Brüne B: Delayed activation of PPARγ in macrophages by LPS and IFN-γ attenuates the oxidative burst. *FASEB J* 15:535–544, 2001
6. von Knethen A, Brüne B: Activation of PPARγ by NO in monocytes/macrophages down-regulates $p47^{phox}$ and attenuates the respiratory burst. *J Immunol* 169:2619–2626, 2002
7. Heinloth A, Brüne B, Fischer B, Galle J: Nitric oxide prevents oxidized LDL-induced p53 accumulation and cytochrome c translocation and apoptosis in macrophages via guanylate cyclase stimulation. *Atherosclerosis* 162:93–101, 2002
8. Semenza GL: HIF-1 and tumor progression: Pathophysiology and therapeutics. *Trends in Mol Med* 8:S62–S67, 2002
9. Brüne B, von Knethen A, Sandau KB: Transcription factors p53 and HIF-1α as targets of nitric oxide. *Cell Signal* 13:525–533, 2001
10. Sandau KB, Fandrey J, Brüne B: Accumlation of HIF-1α under the influence of nitric oxide. *Blood* 97:1009–1015, 2001
11. Sandau KB, Zhou J, Kietzmann T, Brüne B: Regulation of the hypoxia-inducible-factor 1α by the inflammatory mediators nitric oxide and tumor necrosis factor-α in contrast to desferroxamine and phenylarsine oxide. *J Biol Chem* 276:39805–39811, 2001
12. Grisham MB, Jourd'Heul D, Wink DA: Nitric oxide: Physiological chemistry of nitric oxide and its metabolites, implications in inflammation. *Am J Physiol* 276:G315–G321, 1999
13. Brüne B: Nitric oxide (NO) and apoptosis in mesangial cells. *Kidney Int* 61:786–789, 2002
14. Sumbayev V, Sandau KB, Brüne B: Mesangial cells but not Hep G2 hepatocytes are protected towards nitric oxide/superoxide cogeneration: Mechanistic considerations. *Eur J Pharmacol* 444:1–11, 2002