ANTIOXIDANTS & REDOX SIGNALING
Volume 4, Number 5, 2002

Forum Review

Redox Regulation of Cytokine Expression in Kupffer Cells

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ABSTRACT

Kupffer cells, resident macrophages in the liver, play a central role in the homeostatic response to liver injury. Ironically, this defensive mechanism, if dysregulated, also works against the liver in acute and chronic liver damage. Central to this response is activation of nuclear factor-κB (NF-κB), a redox-sensitive transcription factor that transactivates promoters of many inflammatory genes, including cytokines. Much research has been devoted to identification of upstream signaling for activation of NF-κB, but the precise mechanism by which oxidant stress participates in this signaling is yet to be determined. Clues to this key question may be attained through studies on the mechanisms of sustained and/or accentuated NF-κB activation in hepatic macrophages in chronic liver diseases. This article reviews the literature on redox regulation of cytokine gene expression by Kupffer cells. *Antioxid. Redox Signal.* 4: 741–748.

KUPFFER CELLS, TUMOR NECROSIS FACTOR-α (TNAα), AND LIVER DISEASE

KUPFFER CELLS are resident macrophages in the liver, and their population accounts for as much as 70–80% of total body macrophages. They serve to provide the first line of defense in the organ that receives nutrient-rich splanchnic blood and arterial blood totaling up to 25% of cardiac output. These macrophages offer defensive mechanisms against invading microorganisms and neoplasms and function as the major site of endotoxin clearance. They release a wide array of soluble factors, including cytokines, chemokines, growth factors, cyclooxygenase and lipoxygenase metabolites, and gaseous mediators [superoxide anion ($\cdot O_2^-$), hydrogen peroxide (H_2O_2), and nitric oxide ($\cdot NO$)], all of which provide physiologically diverse and pivotal paracrine effects on all other liver cell types. The best example of such effects is induction of acute-phase protein synthesis by hepatocytes, which is mediated by TNFα, interleukin (IL)-1, and IL-6 released by Kupffer cells. Others include regulation of hepatocyte glycogenolysis and oxygen consumption by prostaglandins produced by Kupffer cells. Kupffer cells also play an integral role in liver immune response. They provide the optimal microenvironment for differentiation of hepatic natural killer cells (NK cells) and have intimate and mutually dependent cross-talk with T cells. Kupffer cells are also central to the liver's homeostatic response to injury. Upon degenerative changes in hepatocytes, regardless of whether they are caused by toxicity, infection, or metabolic defects, Kupffer cells immediately respond to an insult and release the mediators to orchestrate inflammatory and reparative responses (Fig. 1). A master initiator of these responses is TNFα, a 17-kDa cytokine released by Kupffer cells. It induces expression of chemokines and adhesion molecules by sinusoidal endothelial cells to initiate recruitment of inflammatory cells. TNFα also induces expression of chemokines and intercellular adhesion molecule-1 (ICAM-1) by hepatic stellate cells (HSC), and this may be a critical event facilitating transmigration of inflammatory cells into the subendothelial (perisinusoidal) space where HSC reside. Inflammatory cells and Kupffer cells recruited into the site of injury work in a consorted fashion to eliminate offending agents, degrade, and remove cellular debris. Matrix remodeling takes place at the same time, and this process is facilitated by matrix metalloproteinase (MMP) released by HSC, inflammatory cells, and Kupffer cells. Kupffer cell-derived TNFα, along with other cytokines, stimulates HSC expression of MMP-2 (72-kDa gelatinase) and MMP-3

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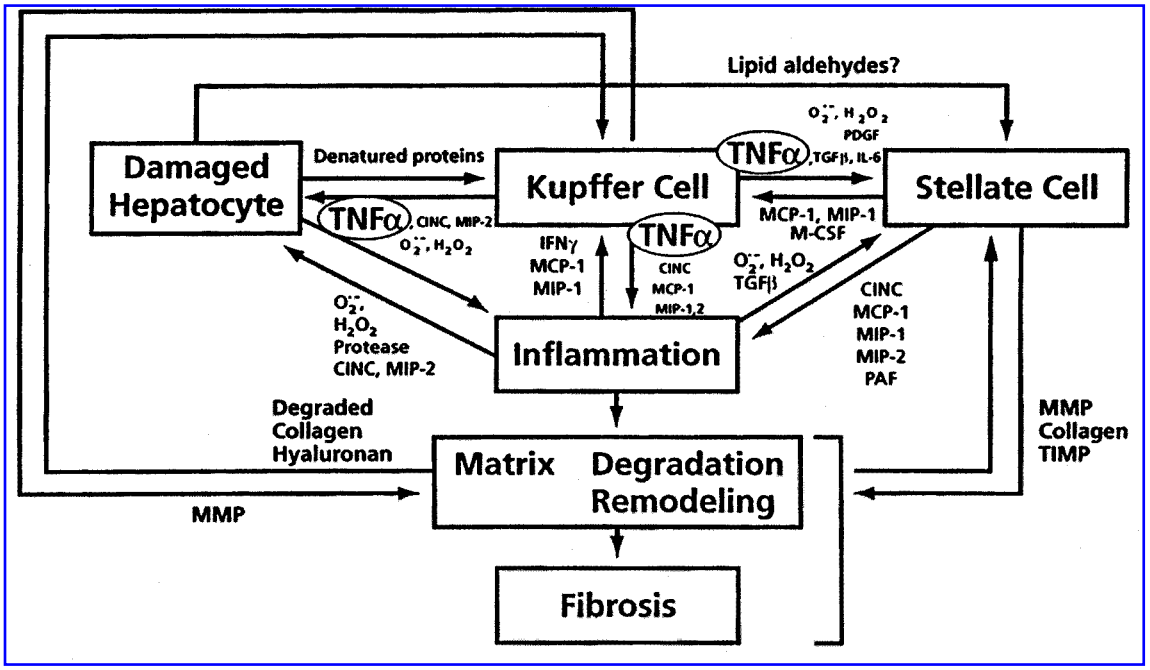

FIG. 1. A schematic diagram depicting the roles of Kupffer cell-derived TNFα in homeostatic responses to liver injury. Note TNFα participates in the initiation of inflammation and matrix remodeling through cellular cross-talk. For the purpose of simplicity, this diagram does not include the important roles played by other nonparenchymal liver cells, such as sinusoidal endothelial cells.

(stromelysin), two key enzymes capable of degrading the components of the perisinusoidal matrices, allowing migration and proliferation of HSC to set a stage for scarring. Thus, the homeostatic responses are initiated by Kupffer cell-derived cytokines at the cellular level and underlie the liver's defense and reparative mechanisms against injury.

It is ironic that the same defensive and protective mechanisms also work against the liver to underlie the pathological process. In fact, compelling evidence demonstrates Kupffer cells' direct role in the pathogenesis of various types of liver injury. The most common approach that investigators have taken to test this hypothesis is to deplete Kupffer cells by injection of gadolinium chloride (2, 5, 11–13, 16, 20, 23, 24, 26, 29, 45) or dichloromethylene diphosphonate as a liposome-encapsulated form (14, 22, 42, 55). Both are preferentially taken up by phagocytic macrophages, particularly Kupffer cells. Therefore, cell death mediated by these toxins is rather selective to Kupffer cells. Using these methods, liver injury induced by numerous hepatotoxic agents, endotoxin, and ischemia–reperfusion was prevented or ameliorated by Kupffer-cell depletion (for review, see 56). Interestingly, most of these pathologic conditions are also ameliorated by blocking TNFα by administration of antibodies or a soluble receptor, or genetic deletion of its receptor. In fact, it is TNFα that is now incriminated in the pathogenesis. Then why is this contradiction of the homeostatic defense response versus the pathogenesis? The answer to this question appears to lie in the quantitative and qualitative differences in expression of TNFα, as well as sensitization of the target cells. At the low level of transient expression seen in acute and mild injury, TNFα serves as a physiological signal to mediate scavenging and reparative processes necessary for reestablishment of the homeostasis. When the expression is intensified or sustained in the case of chronic liver disease or the target cells are sensitized, this pleiotropic cytokine produces excessive or sustained signals to incite cytotoxic and proinflammatory effects (Fig. 2). These effects include the following: (a) cytotoxicity toward hepatocytes via mitochondrial oxidant stress; (b) microcirculation disturbances at hepatic sinusoids due to swelling of endothelial cells and Kupffer cells and hypercoagulative activity; (c) intensified inflammatory responses that further accentuate oxidative injury via activation of NADPH oxidase by inflammatory cells; and (d) excessive matrix remodeling and deposition leading to liver fibrosis and cirrhosis. Thus, the balanced expression of TNFα appears important for liver homeostasis. To this end, the topic of this review

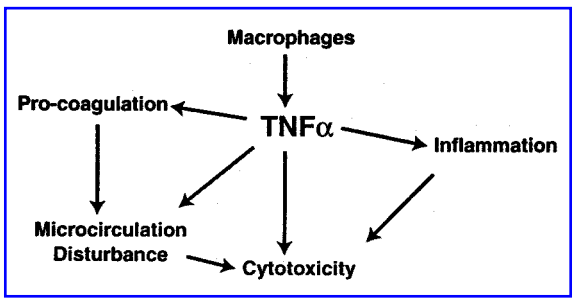

FIG. 2. TNFα can be a key effector molecule for induction of various pathologic conditions in the liver when its expression is sustained or augmented or the target cells become sensitized.

article is highly relevant. Redox regulation is considered most important in expression of TNFα and proinflamamtory mediators by Kupffer cells, and dysregulation of this mechanism potentially causes sustained up-regulation of TNFα as discussed later.

REDOX REGULATION OF GENE EXPRESSION

Before specific research findings on Kupffer cells are discussed, it is important to understand the basic molecular mechanisms we currently know concerning the redox regulation of gene expression. Transcription of certain genes is regulated by oxidative stress or, more specifically, by increased generation of reactive oxygen species (ROS). Classical examples are oxidation-mediated activation of SoxR and OxyR, two redox-sensitive prokaryotic transcription factors that are activated by $\cdot O_2^-$ and H_2O_2, respectively, and induce genes encoding antioxidant enzymes such as catalase, alkyl hydroperoxide reductase, and manganese superoxide dismutase. The redox signal is sensed by an iron-sulfur cluster, [2Fe-2S], in SoxR and a cysteine residue in OxyR. Molecular understanding of these redox-mediated events is incomplete, but the following hypotheses have been proposed. In the case of SoxR, the iron in the [2Fe-2S] cluster in each subunit of the homodimer becomes oxidized, allowing conformational changes in the promoter complex to initiate transcription (18). For OxyR, which does not have a redox-active transition metal, cysteine residues are essential for activation, and this is mediated by oxidation of S-H and formation of an intramolecular disulfide (28, 66). Alternatively or conjunctively, $\cdot NO$ may activate OxyR via *S*-nitrosylation of cysteine residue(s) (17). In fact, *S*-nitrosylation may work through its ability to promote oxidative changes (17) and disulfide formation with the resultant release of $\cdot NO$ (6). Even though these proteins serve as excellent models for understanding the redox regulation of transcription factors, their homologues in eukaryotes have not been identified.

Nuclear factor-κB (NF-κB) is the prototype of the eukaryotic, redox-sensitive transcription factor. NF-κB is a dimeric transcription factor composed of the Rel family of proteins. The Rel family has two major groups. One consists of p50 and p52, which are proteolytically produced from the precursor proteins p105 and p100, respectively. They have a Rel homology domain with the sequences for DNA binding and dimerization and form functional dimers with themselves or a member of another group of Rel proteins comprising Rel A (p65), c-Rel, Rel B, and v-Rel. The latter group of proteins does not have precursor forms and possesses one or more transcriptional activation domains in addition to the Rel homology domain. The most common form of NF-κB is a p50/p65 heterodimer and, unlike other transcription factors, resides in the cytoplasm as its inactive form bound to an inhibitory protein, IκBα.

There are two levels of redox regulation of NF-κB: one in the cytoplasm and another in the nucleus. The former involves phosphorylation of two serine residues (S32 and S36) on IκBα, which results in its polyubiquitination and subsequent degradation by the 26S proteasome, permitting unmasking of the nuclear localization signal and translocation of activated NF-κB into the nucleus. This activation process appears mediated by ROS in lymphoid and monocytic cells, including macrophages. Treatment of the cells with the antioxidant *N*-acetyl-L-cysteine or pyrrolidine dithiocarbamate prevents activation of NF-κB caused by cytokines (48, 51). Addition of H_2O_2 or generation of $\cdot O_2^-$ results in NF-κB activation in certain cell types (3, 48). TNFα-induced activation of NF-κB is abrogated by inhibition of ROS production by the electron transport chain in mitochondria, suggesting oxidant stress from this organelle as a signal for this mode of activation (49). Inhibition of NADPH oxidase blocks or attenuates activation of NF-κB in monocytic cells (10), whereas inhibitors for 5-lipoxygenase blocks both ROS generation and NF-κB activation in lymphoid cells (10), indicating the possible cell type-dependent differences in the source of ROS for activation of this transcription factor. It is important to note that intracellular ROS generation may not be required for NF-κB activation in all cell types. In fact, in several epithelial cell lines, no detectable increase in ROS generation is seen in association with activation of NF-κB (8). Further, antioxidants and metal chelators are often ineffective in blocking NF-κB activation in these cells (9). However, for our discussion of NF-κB activation in macrophages, the role of ROS seems definitely critical.

IκB kinase (IKK) catalyzes phosphorylation of the N-terminal serine residues of IκB to initiate activation of NF-κB. Three IKK subunits have been identified: IKKα and IKKβ as catalytic subunits and IKKγ as a regulatory unit. The most common form of IKK is composed of a heterodimer of IKKα and IKKβ bound to a dimer or trimer of IKKγ. IKK activation occurs when IKKβ is phosphorylated by upstream kinases recruited by IKKγ. This phosphorylation triggers intra- and intermolecular *trans*-phosphorylation of IKK subunits. Much research has been done to identify these upstream kinases. NF-κB inducing kinase (NIK) and mitogen-activated protein/extracellular signal-regulated kinase kinase 1 (MEKK1) are shown to interact directly with and activate IKK (31, 39). NIK mediates IKK activation by double-stranded RNA-activated serine-threonine protein kinase (PKR) (64). Other mitogen-activated protein kinase kinase kinase (MAP3K), such as MEKK2 and MEKK3, were also recently shown to induce IKK activation (65). Thus, it appears that MAP3K and IKK are involved in the signaling for NF-κB activation (Fig. 3). However, these findings are derived from *in vitro* experiments utilizing overexpression of wild-type or mutant kinases, and the *in vivo* roles of these kinases in activation of NF-κB have not been fully addressed. In fact, recent studies using knockout mice demonstrate that MEKK1 is essential for c-Jun N-terminal kinase (JNK) activation, but not for activation of IKK (62), and that NIK is not required for NF-κB activation mediated via TNFα receptor I, but for that induced by lymphotoxinβ receptor (34). Thus, the requirement for upstream kinases for NF-κB activation may be diverse depending on the type of cells and agonists, and further *in vivo* studies are needed to clarify this extremely complex signaling system in a more physiological setting.

A crucial question is how ROS generation leads to IKK activation, particularly in lymphoid and monocytic cells.

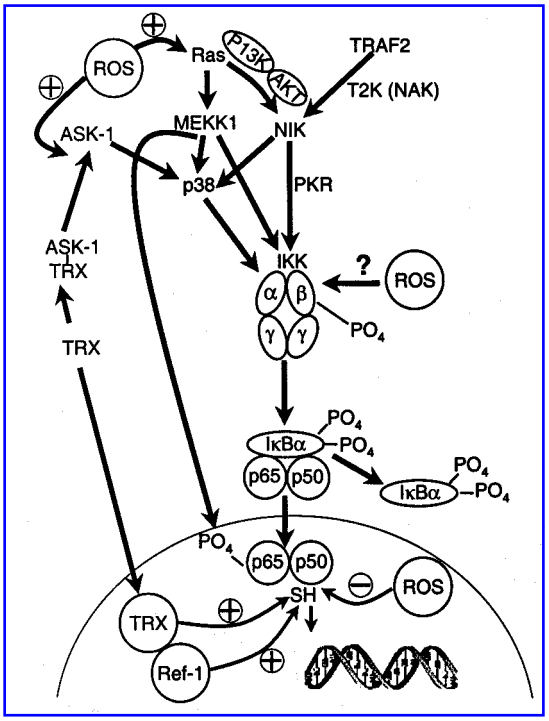

FIG. 3. A diagram depicting proposed complex mechanisms of redox regulation of NF-κB in cytoplasm and nucleus.

Does it have direct effects on IKK or mediate IKK activation via its effects on upstream kinases? For the former, it is possible that cysteine at position 179 within the activation loop of IKKα and IKKβ may be oxidized to form a disulfide bond, bringing the complex in a more tight and stable conformation for phosphorylation of IκBα (47). For ROS-mediated effects on upstream kinases, it is possible a redox-sensitive protein(s) may facilitate activation of such kinases. Thioredoxin (TRX) may be a candidate to fulfill this role. As described later, TRX is capable of reducing cysteine residues of transcription factors in the nucleus to regulate their DNA binding and transactivation activities. But TRX also binds signaling proteins in a redox-sensitive manner. For example, a reduced form of TRX is known to inhibit apoptosis signal-regulating kinase 1 (ASK1), a member of MAP3K family, by binding to its N-terminal portion (44). Upon TNFα stimulation and subsequent generation of ROS, TRX becomes oxidized and releases ASK1, allowing ASK1 to become oligomerized for activation of p38 (32). A similar mechanism of ROS-mediated regulation of MAP3K family members, such as MEKK1 and NIK, may exist (47). ROS may also mediate NF-κB activation in a manner that is independent of nuclear translocation. This is achieved by sequential activation of Ras and mitogen-activated protein kinase, leading to phosphorylation of p65 (41) and consequent augmentation of NF-κB-dependent transactivation (4).

Another major mode of redox-regulation of NF-κB takes place in the nucleus and is direct redox modification of specific cysteine residues in the DNA binding domain of NF-κB. In particular, oxidation of cysteine at position 62 in p50 inhibits DNA binding activity (53). This mode of regulation is not unique to NF-κB, but is relevant to many other transcription factors, including NF-1 (37), SP-1 (61), AP-1 (1), HIF-1 (50), and c-myb (15), which all have redox-sensitive cysteine residues in DNA binding and/or transactivation domains. Here, oxidation inhibits their DNA binding in the nucleus as opposed to oxidative stress-induced activation of NF-κB in the cytoplasm. As part of protective mechanisms for oxidation-mediated suppression of these transcription factors, there are several proteins capable of reducing the critical cysteine residues. These include TRX and (redox factor-1) (Ref-1). Ref-1 is a DNA repair enzyme, but also reduces disulfides in the nucleus and is shown to regulate the redox status and DNA binding activity of NF-κB (36). TRX was recently shown to translocate to the nucleus together with activated NF-κB (21). Overexpression of nuclear-targeted TRX causes increased transactivation of the NF-κB promoter, whereas its overexpression in the cytoplasm results in inhibition of NF-κB activation, suggesting the dual and opposing regulation of NF-κB by TRX at two different sites (21).

KUPFFER CELL NF-KB ACTIVATION

Kupffer cell expression of proinflammatory mediators such as TNFα is largely predicated by transcriptional activation of these genes by NF-κB. Experimental chronic liver injury is frequently associated with sustained activation of NF-κB and induction of TNFα (30, 38, 58). This concept of priming for NF-κB activation in liver disease was originally proposed by McClain and Cohen in 1989, who demonstrated the significantly increased basal and lipopolysaccharide (LPS)-stimulated TNFα release by peripheral blood monocytes from patients with alcoholic hepatitis (35). The same group later confirmed the heightened expression of TNFα and associated enhancement in NF-κB activation in circulating monocytes from these patients (19). Further, the role of oxidative stress was indirectly supported by their demonstration of attenuated NF-κB activation by the *ex vivo* treatment of the cells with various antioxidants (19). This mode of intervention was also implemented to patients with stable alcoholic cirrhosis by treating them with a precursor for glutathione (GSH). This treatment raised the whole blood GSH content in the patients and reduced release of NF-κB-responsive cytokines, TNFα and IL-8 (43). Even though these studies utilized peripheral blood monocytes as a surrogate model for Kupffer cells, a similar observation for redox-sensitive NF-κB activation and regulation of NF-κB responsive genes has been reported in Kupffer cells both *in vitro* and *in vivo*. The antioxidants *N*-acetylcysteine and α-tocopherol inhibit LPS-stimulated Kupffer cell NF-κB activation *in vitro* (7, 19). These antioxidants are also shown to suppress Kupffer cell NF-κB activation *in vivo* in carbon tetrachloride-induced acute liver injury (33) and cholestatic liver injury (30) in rats. An obvious question here again pertains to the source of ROS involved in NF-κB activation in Kupffer cells. As NADPH oxidase represents one major source of ROS as in other inflammatory cells, this multicomponent enzyme is considered important in generating an oxidant signal for activation of NF-κB in the cells. Indeed, in the animal model of

alcoholic liver injury where induction of NF-κB responsive expression of TNFα is pathogenetically critical, the treatment of the animals with diphenyleneiodonium sulfate, a NADPH oxidase inhibitor, blocked free radical generation, NF-κB activation, TNFα expression, and pathology in the liver (27). However, as this inhibitor nonspecifically inhibits flavin-containing oxidases, the proposal needed to be validated via a more definitive approach. For this purpose, mice deficient in $p47^{phox}$, a key cytosolic regulatory component of the NADPH oxidase complex, were used. This study fundamentally reproduced what has been observed in the inhibitor study, confirming the importance of NADPH oxidase-derived ROS in the activation of NF-κB in alcoholic liver injury. NADPH oxidase is located at the plasma membrane and releases abundant ROS extracellularly. Thus, questions arise as to how extracellular generation of ROS leads to intracellular signaling and how much of intracellular ROS is generated by NADPH oxidase activation. For the latter, it is important to note that Rac1, one of the small GTP-binding proteins that belong to the ras superfamily, serves as a regulatory subunit for NADPH oxidase and binds $p67^{phox}$ of the NADPH complex to stimulate the release of $\cdot O_2^-$ in macrophages (40). In fact, transient transfection of a constitutively active mutant of Rac1in HeLa cells results in an increase in intracellular ROS and NF-κB activation in a JNK-independent manner (52). A similar Rac1-mediated $\cdot O_2^-$ generation is also implicated in mitogenic signaling in fibroblasts, but this involves JNK (25). Thus, Rac1 acts as a physiological effector molecule for ROS-mediated signaling within the cell. Indeed, the role of intracellular $\cdot O_2^-$ in NF-κB activation is highlighted by effective amelioration of LPS-induced NF-κB activation in Kupffer cells infected with an adenoviral vector expressing superoxide dismutase (60), and this modality expressing a cytosolic form of superoxide dismutase (CuZnSOD) has been extended to an animal model of alcoholic liver injury in which the pathology was clearly ameliorated (59). These results point to the signaling role of $\cdot O_2^-$ rather than H_2O_2 for NF-κB activation in Kupffer cells and are not in agreement with the previous findings where H_2O_2 was shown to be key in activation of NF-κB and induction of genes driven by this transcription factor (46). More studies are obviously needed to clarify what ROS serves as a signaling molecule in Kupffer cells.

IRON AND KUPFFER CELL NF-κB ACTIVATION

Iron chelator inhibits LPS-induced NF-κB activation in Kupffer cells (30, 58). In fact, the most classical and effective inhibitor of NF-κB, pyrrolidine dithiocarbamate, is a transition metal chelator. Iron has long been implicated in the pathogenesis of chronic liver disease, including alcoholic liver disease. It is believed that iron accumulates in chronic liver inflammation and catalyzes hydroxyl radical-mediated oxidative injury. Indeed, iron chelation ameliorates cholestatic (30) and alcoholic (58) liver injury in animal models, whereas iron supplementation potentiates alcoholic liver injury (57). These studies also demonstrate a tight correlation between the extent of liver injury and NF-κB activation, but we now know that there appears to be a molecular basis for this correlation involving iron-mediated signaling. Kupffer cells isolated from a rat model of alcoholic liver injury have concomitant increases in non-heme iron content, electron paramagnetic resonance-detected generation of radicals, NF-κB binding, and mRNA expression of NF-κB-responsive genes, such as TNFα and macrophage inflammatory protein-1 (MIP-1) (58). Treatment of these cells *ex vivo* with a lipophilic, bidentate iron chelator, deferiprone, normalizes all these parameters. The increased iron content in Kupffer cells appears due to enhanced heme turnover because expression of ferritin and heme oxygenase-1 by Kupffer cells is increased and the splenic content of iron is also raised (58). In support of this notion, the non-heme iron content can be increased in cultured Kupffer cells by erythrophagocytosis, and this promotes LPS-induced NF-κB activation in a heme oxygenase-dependent manner (58). Interestingly, analogous conditions can be observed in foam cells in atherosclerosis known to have the increased iron content (63) or alveolar macrophages that respond to their increased iron content by enhanced production of TNFα and IL-1 (54). Thus, these findings collectively support the role of iron in activation of NF-κB in physiological and pathophysiological settings.

CONCLUSIONS

Compelling evidence exists for the redox regulation of NF-κB activation, and complex upstream signaling events have gradually been elucidated for this regulation in different cell types in response to different agonists. However, critical questions are still actively pursued concerning the source and species of oxidants involved in this process and the precise identity of target molecules of ROS. As exemplified by the rather disappointing recent findings on the roles of NIK (34) and MEKK1 (62) in NF-κB activation *in vivo*, the use of more physiological, *in vivo* models appears pivotal in addressing these specific questions for Kupffer cells in the context of liver biology and pathology.

ACKNOWLEDGMENTS

This work was supported by NIH grants R37AA06603, P50AA11999 (USC–UCLA Research Center for Alcoholic Liver and Pancreatic Diseases), P30DK48522 (USC Research Center for Liver Diseases), and Medical Research Service of Department of Veterans Affairs.

ABBREVIATIONS

ASK, apoptosis signal-regulating kinase; GSH, glutathione; H_2O_2, hydrogen peroxide; HSC, hepatic stellate cells; IKK, IκB kinase; IL, interleukin; JNK, c-Jun N-terminal kinase; LPS, lipopolysaccharide; MAP3K, mitogen-activated protein kinase kinase kinase; MEKK, mitogen-activated protein/extracellular signal-regulated kinase; MIP, macrophage inflammatory protein; MMP, matrix metallopro-

teinase; NF-κB, nuclear factor-κB; NIK, NF-κB inducing kinase; ·NO, nitric oxide; $\cdot O_2^-$, superoxide anion; PKR, RNA-activated serine-threomine protein kinase; Ref-1, redox factor-1; ROS, reactive oxygen species; TNFα, tumor necrosis factor-α; TRX, thioredoxin.

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Received for publication July 11, 2001; accepted April 26, 2002.

This article has been cited by:

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