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Apoptosis of Hepatic Stellate Cells mediated by Specific Protein Nitration

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Short title: Protein nitration in apoptosis of hepatic stellate cells

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Abstract

Inflammatory conditions are characterized by continuous overproduction of nitric oxide (NO) that can contribute to cell survival but also to cell demise by affecting apoptosis. These facts are important in regulation of hepatic fibrogenesis during exposure to inflammatory stress, since elevated NO may pose the risk of cells with a pro-fibrogenic phenotype giving rise to a sustained proliferation leading to chronic fibrosis. Since nitration of tyrosine residues occurs in a range of diseases involving inflammation, we tested the hypothesis that nitration of specific proteins could result in apoptosis of hepatic stellate cells (HSC), the primary cellular source of matrix components in liver diseases. We found the peroxynitrite generator SIN-1 to promote apoptosis in human and rat HSC, based on oligonucleosomal DNA fragmentation, caspase-3 and -9 activation, Bcl-2 depletion and accumulation of Bax protein. We also showed that SIN-1-induced apoptosis of HSC was due to protein nitration. Among the tyrosine-nitrated proteins, tyrosine kinase Lyn was identified. SIN-1 triggered a signaling pathway through Src kinase Lyn activation that resulted in increased activity of the tyrosine kinase Syk. The involvement of these signaling molecules in the apoptotic process induced by SIN-1 as well as the mechanism by which they are activated was confirmed by using specific inhibitors. In summary, NO, via protein-nitration, could play an important role in controlling liver fibrosis resolution by regulation of HSC apoptosis.

Key Words: peroxynitrite; programmed cell death; inflammation; signaling molecules; fibrosis

Abbreviations: NO, nitric oxide; iNOS, inducible nitric oxide synthase; eNOS, endothelial nitric oxide synthase; cNOS, constitutive nitric oxide synthase; SIN-1, 3-morpholinosydnonimide-HCl; Bax, proapoptotic member of the Bcl-2 family of proteins; NF- κ B, nuclear factor kappa B; FeTMPyP, [Iron (III) tetrakis(N-methyl-4'-pyrimidyl)porphyrin-5Cl], PP1, 4-amino-5-(4-methylphenyl)-7-(t-butyl)pyrazolo-d-3,4-pyrimidine); GSH, γ -glutamyl-cysteinyl-glycine.

1. Introduction

The activation of monocytes/macrophages recruited by cytokines and chemotactic factors to inflammation sites is critically important in the development of chronic inflammatory diseases, since these activated cells synthesize and release potentially dangerous molecules, such as nitrogen- and oxygen-derived reactive species [1]. Increased levels of reactive oxygen species are present in different forms of chronic liver injury and are involved in the early phases of hepatic fibrosis, stimulating hepatic stellate cell (HSC) activation, proliferation, invasiveness and collagen production [2,3].

The free radical NO is produced by NO synthases (NOS), which are classified into the constitutive (cNOS) and the inducible (iNOS) isoforms. The constitutively expressed cNOS isoforms are primarily regulated by calcium/calmodulin complexes, whereas active iNOS is rapidly synthesized following immunologic or inflammatory stimuli in several cell types [4]. In addition, cNOS and iNOS differ in the amplitude and duration of NO production, being higher for the iNOS isoform [5,6]. In biological systems, reaction of NO with superoxide forms peroxynitrite, which is more reactive and damaging than its precursor and has been demonstrated to diffuse freely across phospholipid membrane bilayers and to react with a wide variety of molecular targets, including lipids, DNA and proteins [7]. Peroxynitrite has been implicated in a number of pathological situations, and tyrosine nitration induced by this agent has been reported to increase in various human diseases such as acute and chronic inflammatory processes [8].

Liver fibrosis is a common response to chronic liver injury. Activated HSC are the primary cellular source of matrix components in chronic liver disease, and therefore play a critical role in the development and maintenance of liver fibrosis [9,10]. In the normal liver

1 the main function of HSC is storage of vitamin A and other retinoids [11]. However, in
2 response to liver injury HSC become activated and transformed into a proliferative, fibrogenic
3 and proinflammatory myofibroblastic phenotype, characterized by increased expression of α -
4 smooth muscle actin (α -SMA). Activated HSC secrete proteins of the extracellular matrix,
5 such as fibrillar type I and III collagens and fibronectin, and matrix metalloproteinases
6 (MMPs) [12]. Hepatic fibrosis has been shown to be reversible [13] and its resolution requires
7 cessation of activated HSC function [14] which may be achieved via apoptosis [15-17].

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9 Interestingly, excessive NO production by iNOS in the liver has been reported in
10 chronic diseases [18] and NO release induced by pro-inflammatory cytokines has been proven
11 responsible for the decrease of α -SMA, a marker of HSC activation [19]. Moreover, NO
12 donors have been shown to reduce collagen type I deposition in human HSC [20].

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14 Based on the fact that HSC activation often occurs under inflammatory conditions
15 leading to increased NO production, the aim of this study was to investigate the molecular
16 mechanisms involved in the apoptotic effect induced by specific protein nitration in human
17 and rat activated HSC, which could contribute to the resolution of liver fibrosis.

2. Materials and methods

2.1. Reagents

PP1 (Src tyrosine kinase inhibitor), Ac-LEHD-CHO (caspase-9 inhibitor) and Ac-DEVD-CHO (caspase-3 inhibitor) were obtained from Calbiochem (Darmstadt, Germany). Parthenolide (NF- κ B inhibitor), SIN-1 (3-morpholinonydononimide-HCl), piceatannol (non-Src tyrosine kinase Syk inhibitor) and peroxynitrite decomposition catalyst FeTMPyP were supplied by Alexis Biochemicals (San Diego CA). Gallic acid (antioxidant and peroxynitrite scavenger), GSH (endogenous antioxidant) and camptothecin (DNA damaging agent and apoptotic inducer) were purchased from Sigma-Aldrich (St Louis, MO). Cell culture reagents were purchased from Gibco (Grand Island, NY).

2.2. Cell isolation and culture conditions

The cell line of rat hepatic stellate cells (HSC) CFSC-2G was kindly provided by Dr. Rojkind. This cell line was obtained after spontaneous immortalization in culture of HSC isolated from a CCl₄-cirrhotic liver [21], and is characterized by low basal levels of expression of type I collagen genes and by the presence of mRNA for nestin and α -SMA. Therefore, it can be considered as “transitional” HSC, in which the activation process is already initiated. The cell line LX-2 of human HSC was kindly provided by Dr. S. L. Friedman (Mount Sinai School of Medicine, New York). LX-2 are a low-passaged human cell

line derived from normal human HSC spontaneously immortalized [22]. The cells were selected by their ability to grow under low serum conditions (1% fetal bovine serum, FBS).

CFSC-2G cells were cultured in minimum essential medium (MEM; Gibco/BRL, Grand Island, NY) supplemented with 10% FBS and LX-2 cells were cultured in Dulbecco's minimum essential medium (DMEM; Gibco/BRL, Grand Island, NY) supplemented with 1% FBS. Both lines were supplemented with antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin) at 37 °C in a humidified atmosphere containing 5% CO₂. The medium was replaced for serum-free MEM or DMEM for 8 h, after which treatments were carried out.

2.3. Treatments

CFSC-2G and LX-2 cells cultured in serum-free MEM or DMEM were stimulated by adding 250 µM SIN-1 for the indicated times. In some cases cells were pretreated for 30 minutes with PP1 (10 nM), Ac-LEHD-CHO (15-60 µM), Ac-DEVD-CHO (15-60 µM), piceatannol (10 nM), parthenolide (20 µM), gallic acid (10 µM-1 mM), FeTMPyP (10 µM-1 mM), GSH (10 µM-1 mM) or camptothecin (10 µM). Higher concentrations of these compounds were not used to avoid loss of cell viability.

2.4. Determination of oligonucleosomal (histone-associated) DNA fragments

The presence of soluble histone-DNA complexes was measured by the *Cell Death Detection ELISA^{PLUS} 10X* (Roche). For this assay, cells were seeded on 96-well plates at a density of 1×10^5 cells/well. After nonadherent cells were removed, 250 µM SIN-1 was added

1 to the cultures for 8 h, and cell death ELISA assays were performed according to the
2 manufacturers instructions. When indicated, cells were pretreated with different agents in the
3 above described conditions. Specific enrichment of mono- and oligonucleosomes released
4 into the cytoplasm (enrichment factor, EF) was calculated as the ratio between the absorbance
5 values of the samples obtained from treated and control cells.
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11 **2.5. Preparation of cell lysates and Western blot analysis**

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17 Cells were plated on 60-mm diameter culture plates at a density of 6×10^6 cells/plate.
18 After treatment, whole-cell lysates were obtained using 150 μ l of lysis buffer. Protein samples
19 (30 μ g) were size-fractionated by 10% sodium dodecyl sulfate (SDS)-polyacrylamide gels.
20 After electrophoresis, proteins were electrotransferred onto nitrocellulose membranes and
21 blocked for 2 h at room temperature with 1% bovine serum albumin (BSA) or 4% powdered
22 milk in Tris-buffered saline (TBS) containing 0.05% Tween 20 (T-TBS). Blots were
23 incubated overnight at 4 °C with different polyclonal rabbit antibodies: anti-Lyn (Sigma);
24 anti-Bax, anti-Bcl-2, anti-Syk, anti-phospho-Syk, anti-phospho-Src (Cell Signaling); or
25 monoclonal antibodies: anti-nitro-tyrosine (Calbiochem); anti- β -actin (Sigma). Antibodies
26 were diluted according to the manufacture's instructions. After incubation, membranes were
27 extensively washed with T-TBS and incubated for 40 min at room temperature with the
28 appropriate secondary antibody diluted 1:10.000. After washing, blots were developed using
29 the ECL system (Super Signal ULTRA kit, PIERCE) and immunoreactive proteins were
30 visualized on high performance chemiluminescence film (Amersham). Densitometric
31 analyses were carried out with a GS-800 calibrated densitometer (Bio-Rad). Equivalent
32 loading was confirmed using an antibody against actin.
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2.6. Immunoprecipitation

HSC were stimulated with 250 μ M SIN-1 for 30 min. When indicated, cells were pretreated with inhibitors in the above described conditions. Cells were resuspended in an ice-cold lysis buffer. Protein concentration was adjusted to 1 mg/ml in lysis buffer. Anti-nitro-tyrosine antibody (Calbiochem); anti-Lyn (Sigma); anti-phosphotyrosine (Cell Signaling); or anti-Syk (Cell Signaling) was added to each sample and incubated for 2 h at 4 °C. After the addition of 30 μ g of protein A/G PLUS-Agarose (Santa Cruz), an overnight incubation at 4 °C with shaking was carried out. The A/G PLUS-Agarose-beads were pelleted by centrifugation at 12,000 rpm for 2 min at 4 °C and washed four times with lysis buffer at 4 °C to remove nonadsorbed proteins. After the final wash, protein was released from the beads by treatment at 95 °C for 7 min in sample buffer and electrophoresed on 5-10% SDS-PAGE gels. Western blot analysis was completed as described above.

2.7. Measurement of caspase-3 and -9 activities

Caspase-3 and -9 activities were measured in CFSC-2G and LX-2 cells treated for 8 h with 250 μ M SIN-1 using the Caspase-3 and Caspase-9 colorimetric Activity Assay Kit of Sigma and Calbiochem, respectively. When indicated, cells were pretreated with inhibitors in the above described conditions. Cells were scraped in culture medium, pelleted, resuspended in lysis buffer and caspase-3 and -9 activities measured following the manufactures instructions.

2.8. Statistical analysis

The data were analyzed using the Kruskal-Wallis test to determine difference between all independent groups. When significant differences were obtained ($P < 0.05$), differences between two groups were tested using the Mann-Whitney U test.

3. Results

3.1. SIN-1 induces apoptosis of rat and human hepatic stellate cells

In order to study the apoptotic effect of nitric oxide on hepatic stellate cells (HSC), we used SIN-1, a peroxynitrite generator that releases both $\text{NO}^\bullet$ and $\text{O}_2^{\bullet-}$. CFSC-2G and LX-2 cells were treated for 8 h with different concentrations of SIN-1, ranging from 50 to 250 μM . Camptothecin 10 μM was used as a positive control. This DNA damaging agent presents an apoptotic effect mediated by changes in bcl-2 family members and caspase(s) activation. Induction of apoptosis was measured by ELISA as enrichment in histone-associated oligonucleosomal fragments in the cytoplasmic fractions. As shown in figure 1A, SIN-1 exerted on rat and human HSC a dose-dependent apoptotic effect at all the concentrations tested. Time-course experiments were carried out incubating HSC with 250 μM SIN-1 for different time periods, from 2 to 8 h. The levels of cytosolic histone-associated oligonucleosomal fragments were significantly increased after 6 h of treatment in both cell lines (Fig. 1B).

3.2. Molecular mechanisms of SIN-1-induced apoptosis of HSC

The role played by apoptotic regulatory proteins like members of the Bcl-2 family and caspases in SIN-1-induced apoptosis was then investigated in rat and human lines of HSC incubated with 250 μM SIN-1 for 8 h. We analyzed by Western blot protein levels of the anti-apoptotic protein Bcl-2 and the pro-apoptotic protein Bax and we observed decreased levels of Bcl-2 and increased levels of Bax in SIN-1-treated rat and human HSC, as compared to

untreated cells. These effects were abolished by pretreatment of cells with the inhibitor of Src PP1 (Fig. 2).

SIN-1 also increased the activities of caspase-3 and -9 both in CFSC-2G and LX-2 cell lines treated in the same conditions. However, when cells were pretreated with PP1, these effects were not observed (Fig. 3A). In addition, as shown in figure 3B, the apoptotic effect of SIN-1 in both cell lines was prevented in a dose-dependent fashion by a 30 minute pretreatment with 15-60 μ M of either Ac-LEHD-CHO or Ac-DEVD-CHO, inhibitors of caspase-9 and -3, respectively. Unlike these compounds, pretreatment for 30 minutes with 20 μ M parthenolide, a NF- κ B inhibitor, not only did not prevent apoptosis induced by SIN-1, but caused an enhancement on apoptotic cell death both in rat and human HSC.

3.3. Protein tyrosine nitration caused by SIN-1 mediates apoptosis in HSC

We tested the possible nitrating effect of SIN-1 on the Src tyrosine kinase family member Lyn. Analysis of nitrated Lyn levels by Western blot with anti-nitro-tyrosine antibodies of immunoprecipitated extracts obtained using an anti-Lyn antibody was carried out. As shown in figure 4, extracts from cells treated with 250 μ M SIN-1 for 30 minutes presented higher levels of the nitrated form of Lyn compared to control samples, both in rat and human HSC. We also assessed the nitrating effect of SIN-1 on tyrosine residues by analyzing the differential inhibition produced by several compounds: the antioxidant GSH the peroxynitrite decomposition catalyst FeTMPyP, and gallic acid an antioxidant and peroxynitrite scavenger, on SIN-1-induced Lyn tyrosine nitration. The peroxynitrite decomposition catalyst FeTMPyP was the most effective, and the established antioxidant GSH, used for comparative purposes, was found to be less effective than the other agents (Fig. 4).

In other experiments we established the involvement of protein nitration in the apoptotic effect of SIN-1 using the same agents: GA, FeTMPyP and GSH. All compounds diminished in a dose-response fashion the apoptotic effect of SIN-1 determined as above, FeTMPyP being also the most effective (Fig. 5). These results indicate that, among other possible oxidative effects, SIN-1 induces apoptosis in both cell lines by causing protein nitration.

3.4. Role of Lyn and Syk on SIN-1-induced apoptosis of HSC

Since SIN-1 has been described to modulate the activity of some tyrosine kinases, the effect of this compound on tyrosine phosphorylation of Lyn and Syk was analyzed in rat and human HSC pretreated or not with PP1 or FeTMPyP, inhibitors of Src kinases and peroxynitrite, respectively. We tested the effect of SIN-1 on the phosphorylation of the Src family tyrosine member Lyn and the non-Src tyrosine kinase, Syk. Tyrosine phosphorylation levels of Lyn and Syk were determined by Western blot analysis of the phosphorylated forms of immunoprecipitated extracts obtained with antibodies specific against both kinases. Figure 6 shows that treatment of human and rat HSC with 250 μ M SIN-1 for 30 minutes induced tyrosine phosphorylation of Lyn and Syk respectively, and pretreatment with PP1 or FeTMPyP diminished these effects.

The functional role of Lyn and Syk in SIN-1-induced apoptosis was evaluated by using the respective inhibitors for these tyrosine kinases, PP1 and piceatannol. Rat and human HSC were pretreated with 10 nM of each inhibitor for 30 minutes, and then treated with 250 μ M SIN-1 for 8 h. We observed that SIN-1-induced apoptosis of HSC was clearly prevented by inhibition of Lyn or Syk activities (Fig. 7).

4. Discussion

The free radical gas NO synthesized by different NOS isoforms, has been shown to have many physiologic and pathophysiologic functions being a potent mediator in cardiovascular, peripheral and central nervous systems and a key element of the immune response [23,24].

The main isoforms of NOS in the liver are eNOS (endothelial NO synthase) and iNOS [25]. Nitric oxide is released in the liver by different cell types including endothelial cells, macrophages, hepatocytes and Kupffer cells in response to several stimuli. Under normal physiologic conditions, NO is generated constitutively from sinusoidal eNOS. However, in liver injury eNOS activity has been shown to be markedly reduced whereas iNOS activity is significantly elevated with concomitant HSC activation [26]. iNOS could be induced in monocyte/macrophages, Kupffer cells and hepatocytes by inflammatory cytokines [4,27,28]. Increased production of NO by iNOS and reactive oxidants derived from nitric oxide has been proposed to mediate tissue injury in several inflammatory and infectious diseases including hepatic diseases [7,18,19]. NO is able to trigger its own biosynthesis through iNOS induction in macrophages but not in hepatocytes, generating a potent amplification mechanism that may constitute the basis for the excessive formation of NO in acute and chronic inflammatory conditions [4,29].

Whereas it is clear that eNOS-derived NO production is protective in the liver, there are contradictory results regarding to the nature of the effect of NO generated by iNOS [20, 30,31], that could be partially due to differences in the intensity and duration of injury [5,6]. In addition, interactions between NO and reactive oxygen species (ROS) are also important in the ultimate effects of NO. Although NO can inhibit the generation of oxygen radicals and

1 decrease lipid peroxidation, cytotoxic actions of NO can occur at high levels of ROS as a
2 consequence of its transformation to peroxynitrite or other derived reactive species. Some
3 reports indicate that peroxynitrite in fact induces cell death in a variety of cell types, and that
4 the apoptotic effect of NO could be mediated, at least in part, by peroxynitrite-mediated
5 nitration of some key proteins triggering apoptosis [32-34]. In the liver simultaneous
6 production of NO and superoxide by resident Kupffer cells and infiltrating macrophages may
7 generate peroxynitrite, which leads to protein nitration and apoptosis in different types of
8 hepatic cells by acting in autocrine and paracrine fashions [35,36].

19 Assuming that SIN-1 can simultaneously generate superoxide and nitric oxide which
20 results in peroxynitrite formation [37], this compound appears as a useful tool to study the
21 role played by protein nitration in peroxynitrite-induced apoptosis of HSC, although other
22 oxidative changes cannot be ruled out. In this study we showed that SIN-1 caused a
23 significant apoptotic effect on HSC in a concentration-dependent fashion (10-250 μ M). The
24 involvement of the mitochondrial cell death pathway in the proapoptotic effect of NO is
25 generally accepted. In this pathway, pro- and anti-apoptotic members of the Bcl-2 family
26 regulate the release of cytochrome c to associate with Apaf-1 in order to form a multimeric
27 complex that recruits and activates procaspase-9. This initiator caspase causes then proteolytic
28 activation of the executioner caspase-3, leading to the characteristic morphological and
29 biochemical features of apoptosis. We showed that incubation of HSC with SIN-1 resulted in
30 decreased Bcl-2 and increased Bax protein levels, involving this protein family in SIN-1-
31 induced apoptosis of HSC. The elevated Bax/Bcl-2 ratio so achieved would then induce the
32 activation of caspases-9 and -3 leading to subsequent oligonucleosomal fragmentation, as we
33 have also observed. Previous reports suggest that NO promotes caspase-independent HSC
34 apoptosis probably mediated by nitrosylation of some cellular components [38]. The present
35 results indicate that NO can also induce caspase-dependent apoptosis of HSC mediated by

1 protein nitration via the peroxynitrite produced by hepatic cells, and more importantly by
2 infiltrating macrophages in inflammatory conditions [39,40]. In addition, it is established that
3 infiltrating macrophages not only participate in the development of fibrosis but are also
4 pivotal in fibrosis resolution [41,42]. Apoptosis of HSC involving different caspases has also
5 been shown to be induced by superoxide and other agents [43]. We also found that inhibition
6 of NF- κ B caused apoptosis by itself and enhanced SIN-1-induced apoptosis in HSC,
7 suggesting a protective effect of NF- κ B in this process, in agreement with other studies [44].

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9 The effects of SIN-1 on Bax/Bcl-2 ratio and caspases-9 and -3 activities were
10 prevented by pre-treatment with the Src inhibitor PP1, indicating that some member(s) of this
11 family of kinases, which are essential integrators of signaling events [45], regulate these
12 apoptotic effectors. It is also known that nitration may affect the phosphorylation of tyrosine
13 residues in some proteins, promoting phospho-tyrosine signaling in a variety of cell types
14 [46]. Activation of Src kinases by peroxynitrite has been previously demonstrated, and it has
15 been also proposed that nitration of tyrosine residues can mediate the activation of the Src
16 kinase Lyn [47]. In the present work we were able to show SIN-1-induced nitration of Lyn
17 kinase in HSC which correlated with an increase in the activating phosphorylation of this
18 protein. Also Syk, a described substrate of Src kinases including Lyn [48,49], was
19 phosphorylated in response to treatment of cells with peroxynitrite in a Src kinase activity-
20 dependent manner. Although the multiple functions of Src and Syk families in hematopoietic
21 cells [50] are well established, little is known about them in hepatic cells as the present report
22 is the first describing both the presence and function of Syk in rat and human HSC. Indeed,
23 we assessed that signaling events involving Src and Syk kinases (figure 8) could mediate SIN-
24 1-induced apoptosis in HSC by demonstrating that the apoptotic effect of this compound was
25 prevented by specific inhibitors for these signaling molecules.

1 Taking into account that the resolution phase of fibrosis is now clear to require loss of
2 activated HSC via apoptosis, our results suggest that, among other negatively acting
3 regulatory pathways, peroxynitrite-induced nitration of Lyn could mediate recovery from
4 hepatic fibrosis by triggering apoptosis of activated HSC, thus contributing to elimination of
5 these potentially dangerous cells. Although this hypothesis should be confirmed in animal
6 models for liver injury in order to be relevant for clinical approaches, the possible beneficial
7 effect of peroxynitrite should be taken into account in the design of anti-fibrogenic therapies
8 in response to different kinds of liver damage.
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Legends

Figure 1. Apoptosis induced by SIN-1 in rat and human HSC. (A) Dose-response analysis of apoptosis in immortalized non-tumoral rat (CFSC-2G) and human (LX-2) HSC cell lines treated with SIN-1. Cells were treated for 8 h with SIN-1 at concentrations ranging from 50 to 250 μ M, or with camptothecin (CPT) 10 μ M, used as a positive control. (B) Time course analysis of apoptosis induced by SIN-1 in rat and human HSC. Cells were treated for 2-8 h with 250 μ M SIN-1. Oligonucleosomal fragments content was analyzed by ELISA and expressed as enrichment factor (EF), as described in Materials and Methods. Data are the average $\pm$ standard deviation of values from five independent experiments. Statistical analysis was carried out by comparing the data with values from untreated cells using the Mann-Whitney U test (** $p < 0.01$).

Figure 2. Western blot analysis of Bcl-2 and Bax protein levels in SIN-1-treated HSC. CFSC-2G and LX-2 cells were incubated with or without SIN-1 (250 μ M) for 8 h in the presence or absence PP1 (Src kinase inhibitor). Protein levels of Bcl-2 and Bax were detected by Western blot using specific antibodies. Actin levels were used as a loading control. Upper panel, representative Western blot of five independent experiments; lower panel, fold change of total Bcl-2 or Bax levels normalized to actin determined by densitometry (** $p < 0.01$; *a*, vs control; *b*, vs SIN-1-treated cells).

Figure 3. Role of caspase-3 and -9 in SIN-1-induced apoptosis of HSC. (A) Analysis of caspase-3, and -9 activities in SIN-1-treated HSC. Rat (CFSC-2G) and human (LX-2) HSC were incubated with or without SIN-1 (250 μ M) for 8 h in the presence or absence of PP1 (Src kinase inhibitor). Caspase-3 and -9 activities were analyzed by colorimetric Activity Assay Kits of Sigma and Calbiochem, respectively, as described in Materials and Methods. The results are representative of at least five independent experiments (** p < 0.01; *a*, vs control; *b*, vs SIN-1-treated cells). (B) Effect of caspase inhibitors on SIN-1-induced apoptosis of rat and human HSC. HSC were incubated with or without 250 μ M SIN-1 for 8 h. When indicated, cells were pretreated with 15-60 μ M Ac-LEHD-CHO (caspase-9 inhibitor) or Ac-DEVD-CHO (caspase-3 inhibitor), or with 20 μ M parthenolide (PAR, NF- κ B inhibitor). Oligonucleosomal fragments content was measured as described in Materials and Methods expressed as enrichment factor (EF). Data are the average $\pm$ standard deviation of values from five independent experiments. Statistical analysis was carried out by comparing the data with their respective controls by using the Mann-Whitney U test (* p < 0.05, ** p < 0.01; *a*, vs control; *b*, vs SIN-1-treated cells).

Figure 4. Analysis of Lyn nitration in response to SIN-1. CFSC-2G and LX-2 cells were incubated with or without 250 μ M SIN-1 for 30 min, and when indicated pretreated with peroxynitrite decomposition catalyst FeTMPyP (FE), antioxidant and peroxynitrite scavenger gallic acid (GA) or antioxidant glutathione (GSH, reduced form), 100 μ M for 30 min. Upper panel, representative Western blot analysis of nitrated Lyn using specific anti-nitro-tyrosine antibodies in protein extracts immunoprecipitated with antibodies against Lyn; lower panel, fold change of Lyn nitration determined by densitometry (** p < 0.01; *a*, vs control; *b*, vs SIN-1-treated cells). Blots are representative of at least five independent experiments.

Figure 5. Effect of gallic acid, FeTMPyP and reduced glutathione on SIN-1-induced apoptosis of HSC. Rat (CFSC-2G) and human (LX-2) HSC were incubated with or without 250 μ M SIN-1 for 8 h. When indicated, cells were pretreated for 30 min with antioxidant and peroxynitrite scavenger gallic acid (GA), peroxynitrite decomposition catalyst FeTMPyP (FE), or antioxidant glutathione (GSH, reduced form), (10 μ M-1mM). Oligonucleosomal fragments content was measured as described in Materials and Methods expressed as enrichment factor (EF). Data are the average $\pm$ standard deviation of values from five independent experiments. Statistical analysis was carried out by comparing the data with their respective controls by using the Mann-Whitney U test (* p < 0.05, ** p < 0.01; *a*, vs control; *b*, vs SIN-1-treated cells).

Figure 6. Analysis of Lyn and Syk phosphorylation in response to SIN-1. CFSC-2G and LX-2 cells were incubated with or without 250 μ M SIN-1 for 30 min, and when indicated pretreated with 10 nM Src kinase inhibitor PP1 or 100 μ M peroxynitrite decomposition catalyst FeTMPyP (FE) for 30 min. Upper panels, representative Western Blot analysis of phosphotyrosine levels in protein extracts immunoprecipitated with antibodies against Lyn (A) or Syk (B) using specific anti-phospho-Src (A) or anti-phospho-Syk (B) antibodies; lower panels, fold change of Lyn or Syk phosphorylation determined by densitometry (** p < 0.01; *a*, vs control; *b*, vs SIN-1-treated cells). Blots are representative of at least five independent experiments.

Figure 7. Role of Lyn and Syk activities on SIN-1-induced apoptosis of HSC. Rat (CFSC-2G) and human (LX-2) HSC were incubated with or without 250 μ M SIN-1 for 8 h.

When indicated, cells were pretreated with 10 nM of Src kinase inhibitor PP1 or Syk inhibitor piceatannol (PICEAT). Oligonucleosomal fragments content was measured as described in Materials and Methods expressed as enrichment factor (EF). Data are the average $\pm$ standard deviation of values from five independent experiments. Statistical analysis was carried out by comparing the data with their respective controls by using the Mann-Whitney U test (* $p < 0.05$, ** $p < 0.01$; *a*, vs control; *b*, vs SIN-1-treated cells).

Figure 8. Proposed model for peroxynitrite-mediated apoptosis induced by NO in HSC.

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*Graphical Abstract