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DLPC and SAMe prevent α1(I) collagen mRNA up-regulation in human hepatic stellate cells, whether caused by leptin or menadione

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Abstract

We previously reported that the combination of dilinoleoylphosphatidylcholine (DLPC) and *S*-adenosylmethionine (SAMe), which have antioxidant properties and antifibrogenic actions, prevented leptin-stimulated tissue inhibitor of metalloproteinase (TIMP)-1 production in hepatic stellate cells (HSCs) by inhibiting H_2O_2-mediated signal transduction. We now show that DLPC and SAMe inhibit α1(I) collagen mRNA expression induced by leptin or menadione in LX-2 human HSCs. We found that DLPC and SAMe prevent H_2O_2 generation and restore reduced glutathione (GSH) depletion whether caused by leptin or menadione. Blocking H_2O_2 signaling through ERK1/2 and p38 pathways resulted in a complete inhibition of leptin or menadione-induced α1(I) collagen mRNA. The inhibition of collagen mRNA by DLPC and SAMe combined is at least two times more effective than that by DLPC or SAMe alone. In conjunction with the prevention of TIMP-1 production, the ability of DLPC and SAMe to inhibit α1(I) collagen mRNA expression provides a mechanistic basis for these innocuous compounds in the prevention of hepatic fibrosis, because enhanced TIMP-1 and collagen productions are associated with hepatic fibrogenesis and their attenuation may diminish fibrosis.

Keywords: Dilinoleoylphosphatidylcholine; *S*-Adenosylmethionine; Leptin; Menadione; Hepatic stellate cells; α1(I) collagen mRNA; H_2O_2; Reduced glutathione; ERK1/2; p38

We previously reported that hepatic stellate cells (HSCs) produce the oxidant H_2O_2 in response to the fibrogenic hormone leptin [1–3]. These studies emphasized the central role of H_2O_2 in the activation of extracellular signal-regulated kinase (ERK)1/2 and p38 mitogen-activated protein kinase (MAPK) signaling pathways which mediate expression of fibrogenic genes, including α1(I) procollagen, tissue inhibitor of metalloproteinase (TIMP)-1, and matrix metalloproteinase (MMP)-1. Because these are the major determinants of hepatic fibrogenesis, it appears that blocking H_2O_2 production in HSCs with antioxidants would be beneficial against HSC fibrogenesis. Accordingly, we showed that inhibition of H_2O_2 by dilinoleoylphosphatidylcholine (DLPC) and *S*-adenosylmethionine (SAMe), which have antioxidant properties and antifibrogenic actions, resulted in down-regulation of TIMP-1 gene and attenuation of TIMP-1 protein secretion in HSC culture after leptin treatment [4]. The prevention of TIMP-1 production by DLPC and SAMe is attributable, at least in part, to inhibition of H_2O_2-dependent ERK1/2 and p38 signaling pathways. We now wondered whether inhibition of H_2O_2 also results in down-regulation of α1(I) procollagen mRNA, a commonly used marker of liver fibrogenesis. To test this hypothesis, H_2O_2 was generated in HSCs using two model compounds, namely leptin, as shown before [1–4], and menadione (2 methyl-1,4-naphthoquinone), an avid generator of H_2O_2 in liver cells [5]. These were investigated in a human HSC line, LX-2, which retains key features of activated HSCs [1,6].

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Materials and methods

Culture and treatment of HSCs. LX-2 cell line, derived from human HSCs [6], was kindly provided by Dr. Scott L. Friedman, Mount Sinai School of Medicine, NY. The cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 5% heat-inactivated fetal calf serum as previously described [1]. At subconfluence, cells were washed in serum-free DMEM and then incubated in the same media containing leptin or menadione in the absence or presence of DLPC, SAMe or various inhibitors (see below) for various intervals of time. Leptin (Sigma Chemicals, St. Louis, MO), dissolved in DMEM, was used at 25–100 ng/ml. Menadione (Sigma) was dissolved in dimethyl sulfoxide (DMSO), and was used at 15, 30, and 45 μM. DLPC (Avanti Polar Lipids, Alabaster, AL), dissolved in 0.05% bovine albumin, was used at 10 μM, and SAMe (Sigma) dissolved in saline, at 12 μM [4]. Other inhibitors and their concentrations were: 1000 U/ml catalase [1] (Sigma); 30 μM ERK1/2 inhibitor PD098059 [7] (Sigma), and 20 μM p38 inhibitor SB203580 [8] (Sigma). Catalase was dissolved in the culture media, and PD098059 and SB203580 were dissolved in DMSO. In these experiments, LX-2 cells were used at passages 20–30.

α1(I) collagen mRNA assay. Expression of mRNA for the α1(I) collagen in LX-2 cells was evaluated by Northern blots as previously described [2]. The levels of the mRNA were quantified by measuring the intensity of the bands on X-ray film by imaging densitometry.

Intracellular H_2O_2 and reduced GSH determination. H_2O_2 in LX-2 cell lysates was quantified using the H_2O_2 Assay Kit (Cayman Chemical, Ann Arbor, MI). The procedure was based on the oxidation of ferrous ions to ferric ions by H_2O_2 under acidic conditions. The ferric ions bind with the dye xylenol orange to form a stable colored complex which is measured at 595 nm. Catalase which decreased H_2O_2 to an undetectable level served as control. GSH in LX-2 cells was measured using the GSH Assay Kit (Cayman) according to the manufacturer's instructions.

ERK1/2 and p38 MAPK phosphorylation assays. The levels of MAPK phosphorylation were determined at 2 h, the time shown to be associated with maximal phosphorylation of ERK1/2 and p38 by leptin in LX-2 cells [1]. Phosphorylated (p)-ERK1/2 and p-p38 proteins were analyzed by Western blots, using the PhosphoPlus ERKl/2 MAPK and p38 MAPK Antibody Kits (Cell Signaling Technology, Beverly, MA) as previously described [1]. ERK1/2 phosphorylation was detected using a rabbit phospho-ERK1/2 (Thr-202/Tyr-204) antibody and p38 phosphorylation, using a rabbit phospho-p38 (Thr-180/Tyr-182) antibody. Signal intensities were quantified by imaging densitometry.

Statistics. Data are reported as means ± SE and the significance of difference between means was assessed using analysis of variance followed by Student–Neuman Keuls tests. A value of $p < 0.05$ was considered to be significant.

Results

Inhibition of leptin-induced α1(I) collagen mRNA by DLPC, SAMe, and their combination

The Northern blots in Fig. 1A revealed two transcripts of α1(I) collagen mRNA (5.8 and 4.8 kb) expressed by LX-2 cells, which were similar to that observed for primary human cultured HSCs [9]. Leptin enhanced the mRNA levels in a dose-dependent manner with a 3.5-fold increase at 75 ng/ml. Both DLPC (10 μM) and SAMe (12 μM) alone decreased the rise by 42% and 34%, respectively (Fig. 1B). The combination of DLPC and SAMe fully inhibited collagen mRNA up-regulation, and the effect was significantly different from that of DLPC or SAMe alone ($p < 0.001$).

Inhibition of menadione-induced α1(I) collagen mRNA by DLPC, SAMe, and their combination

Fig. 2A shows that menadione treatment of LX-2 cells up-regulated α1(I) collagen mRNA. Of the 3 doses of menadione tested (15, 30, and 45 μM), the 30 one produced the greatest increase of the mRNA, at 3.7-fold over the control. At this dose of menadione, no cell toxicity of HSCs was observed, and it was used in subsequent experiments. DLPC or SAMe alone halved the menadione-stimulated collagen mRNA, whereas DLPC and SAMe combined

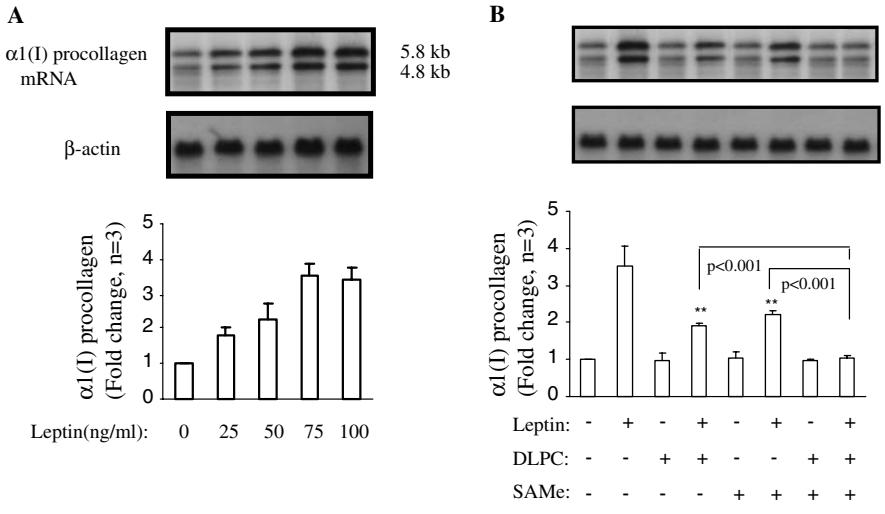

Fig. 1. Leptin stimulates mRNA expression α1(I) collagen and its inhibition by DLPC and SAMe. (A) Effect of leptin concentrations. LX-2 cells were incubated with various concentrations of leptin for 24 h. Upper panels are representative Northern blots showing two mRNA transcripts for the α1(I) collagen gene. The lower panel shows the corresponding histograms of data of three separate analyses. The mRNA levels were normalized to β-actin, and values are expressed as fold change relative to control (no leptin) assigned a value of 1. (B) Inhibition by DLPC and SAMe of collagen mRNA. LX-2 cells were incubated with leptin (75 ng/ml) in the presence or absence of DLPC (10 μM) or SAMe (12 μM) or DLPC + SAMe for 24 h. Upper panels are representative Northern blots and the histograms in the lower panel summarizing the data of three separate analyses. Values are expressed as fold change relative to control (no leptin, DLPC or SAMe). $^{**}p < 0.01$ vs. leptin without DLPC and SAMe.

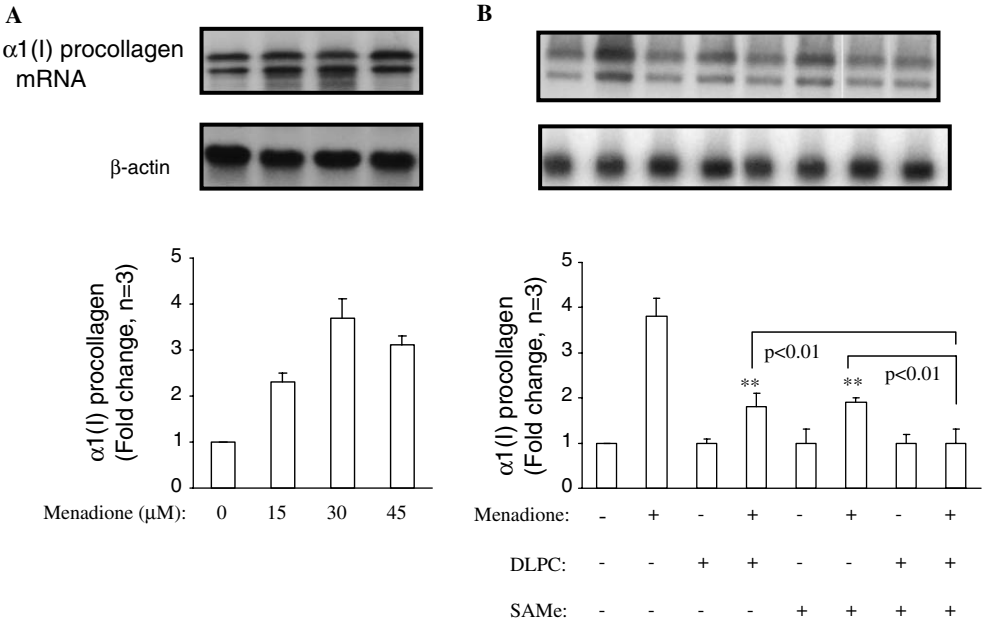

Fig. 2. Menadione stimulates α1(I) collagen mRNA expression and its inhibition by DLPC and SAMe. (A) Effect of menadione doses. LX-2 cells were incubated with menadione at the indicated doses for 24 h. Upper panels are representative Northern blots showing two mRNA transcripts for the α1(I) collagen gene. The lower panel shows the corresponding histograms of data of three separate analyses. The mRNA levels were normalized to β-actin, and values are expressed as fold change relative to control (no menadione) assigned a value of 1. (B) Inhibition by DLPC and SAMe of collagen mRNA. LX-2 cells were incubated with menadione (30 μM) in the presence or absence of DLPC (10 μM) or SAMe (12 μM) or DLPC + SAMe for 24 h. Upper panels are representative Northern blots and the histograms in the lower panel summarize the data of three separate analyses. Values are expressed as fold change relative to control (no menadione, DLPC or SAMe). $^{**}p < 0.01$ vs. menadione without DLPC and SAMe.

completely prevented the up-regulation (Fig. 2B), and the effect of DLPC + SAMe was significantly different from that of DLPC or SAMe alone ($p < 0.01$).

Effect of catalase on α1(I) collagen mRNA up-regulation by leptin or menadione

Fig. 3A and B show that catalase, an antioxidative enzyme and an H_2O_2 scavenger, completely blocked leptin or menadione up-regulation of collagen mRNA, suggesting that H_2O_2 is involved in the induction of the mRNA, whether by leptin or menadione.

Leptin and menadione enhance H_2O_2 production and diminish reduced GSH; the effects are opposed by DLPC + SAMe and catalase

To demonstrate that H_2O_2 is indeed generated in LX-2 cells, we measured the concentration of the peroxides after leptin or menadione treatment (Table 1). The intracellular level of H_2O_2 was raised 10 times after leptin and menadione. DLPC and SAMe combined diminished H_2O_2 production by leptin and menadione by 72% and 70%, respectively, reflecting their antioxidant properties. Catalase abolished the H_2O_2 increase after leptin or menadione, in accordance with its H_2O_2 scavenger action.

The data in Table 1 also show that reduced GSH was diminished by 36% after leptin and 43% after menadione. DLPC + SAMe restored the respective GSH decreases to

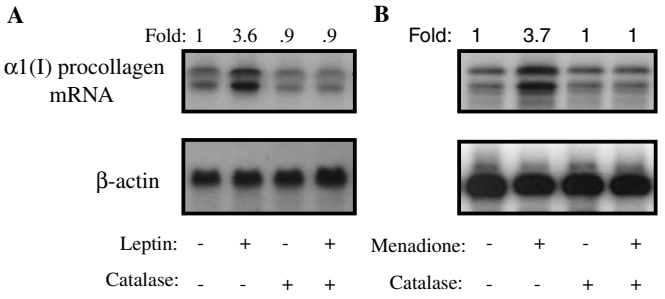

Fig. 3. Catalase prevents α1(I) collagen mRNA expression whether induced by leptin or menadione. LX-2 cells were treated with leptin (75 ng/ml) in (A) and menadione (30 μM) in (B) in the presence of catalase (1000 U/ml) or its absence. The mRNA levels were analyzed by Northern blots 24 h after the treatment. Representative blots of three separate analyses were shown and values (numbers above the blots) are expressed as fold relative to the controls assigned a value of 1: no leptin and catalase in (A) and no menadione and catalase in (B).

more than 90% of the value of control cells. Catalase fully normalized the GSH decrease after leptin or menadione, suggesting an involvement of H_2O_2 in the depletion of GSH.

DLPC and SAMe combined and catalase prevent phosphorylation of ERK1/2 and p38 induced by leptin and menadione

To evaluate whether the antioxidant action of DLPC and SAMe, and catalase against H_2O_2 formation results

Table 1
H_2O_2 and reduced glutathione (GSH) in LX-2 cells

Control	Leptin	DLPC + SAMe	Leptin DLPC + SAMe	Menadione	Menadione DLPC + SAMe	Catalase	Leptin catalase	Menadione catalase
H_2O_2 (μM/mg protein)								
2.4 ± 0.4	$23.9 \pm 2^{*}$	2.3 ± 0.3	$6.8 \pm 2.5^{**}$	$24.8 \pm 1.7^{*}$	$7.5 \pm 3^{***}$	2.3 ± 0.1	1.5 ± 0.1	$1.4 \pm 0.1^{***}$
GSH (nM/mg protein)								
13.1 ± 2.1	$8.4 \pm 2^{*}$	12.6 ± 2.3	$12.5 \pm 2.5^{**}$	$7.5 \pm 1^{*}$	$11.9 \pm 2^{***}$	12.1 ± 1.6	$13.2 \pm 2.1^{**}$	$13.8 \pm 1.6^{***}$

H_2O_2 and GSH were quantified after 1 h of treatment using the Cayman H_2O_2 Assay Kit and GSH Assay Kit, respectively. Data are means ± SEM, $n = 5$.
* $p < 0.001$ vs. control.
** $p < 0.001$ vs. leptin.
*** $p < 0.001$ vs. menadione.

in inhibition of ERK1/2 and p38 signal transduction, we determined phosphorylation of ERK1/2 and p38. Leptin increased the phosphorylation of ERK1/2 4.1-fold and that of p38, 3.4-fold (Fig. 4A). DLPC and SAMe combined brought the respective increases down to nearly the control levels. Menadione enhanced phosphorylation of ERK1/2 3.6-fold and of p38, 3.1-fold; these increases were fully prevented by DLPC + SAMe (Fig. 4B), and, similarly, by catalase (Fig. 4C). These results suggest that DLPC and SAMe inhibit ERK1/2 and p38 phosphorylation by an H_2O_2-dependent mechanism similar to that of catalase.

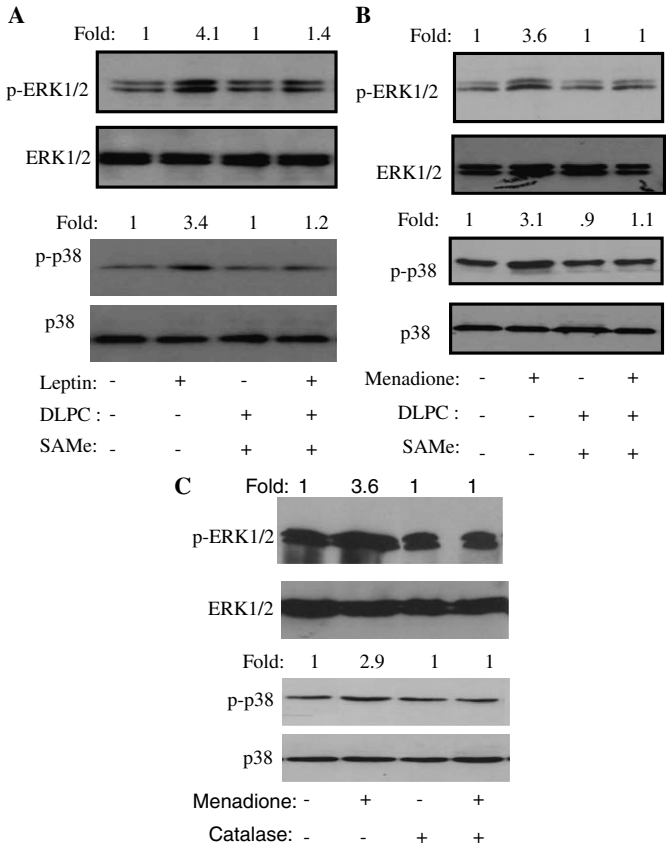

Fig. 4. Increased phosphorylation of ERK1/2 and p38 after leptin or menadione is prevented by DLPC + SAMe or catalase. LX-2 cells were incubated with leptin (A) and menadione (B) in the presence of DLPC + SAMe or in their absence. The levels of p-ERK1/2 and p-p38 were determined by Western blots after 2 h of the treatment. The intensity of the bands on the blots was normalized to that of total ERK1/2 or total p38. The number above the blots are values of three separate analyses, expressed as fold relative to controls assigned a value of 1: no leptin, DLPC and SAMe in (A), and no menadione, DLPC, and SAMe in (B). (C) Cells were treated with menadione in the absence or presence of catalase (1000 U/ml), and ERK1/2 and p38 phosphorylation was determined by Western blots 2 h after the treatment.

Blockade of ERK1/2 and p38 phosphorylation result in inhibition of α1(I) collagen mRNA stimulated by leptin or menadione

Both the ERK1/2 inhibitor PD098059 and p38 inhibitor SB203580 reduced the 3.6-fold increase of collagen mRNA after leptin to nearly one-half (Fig. 5A). The 3.7-fold stimulation of the mRNA by menadione was halved by PD098059 and attenuated 42% by the p38 inhibitor SB203580 (Fig. 5B). These results demonstrate that the ERK1/2 and p38 signaling pathways contribute almost equally to α1(I) collagen mRNA up-regulation, whether induced by leptin or menadione.

Discussion

This study revealed that the combination of DLPC and SAMe prevents H_2O_2 production in LX-2 HSCs, whether it was caused by leptin or menadione. This effect resulted in down-regulation of α1(I) collagen mRNA through inhibition of the H_2O_2-dependent ERK1/2 and p38 signal transduction. The inhibition of collagen mRNA expression afforded by DLPC and SAMe combined is at least two times more effective than that by DLPC or SAMe alone.

We report here for the first time the ability of menadione to induce mRNA expression of α1(I) collagen in HSCs. At the dose of 30 μM, which was not cytotoxic to the HSC line (data not shown), menadione stimulated collagen mRNA 3.5-fold, an effect which equaled that of leptin (75 ng/ml), a hormone with fibrogenic action in the liver [10]. Like leptin, menadione used the ERK1/2 and p38 signaling pathways to up-regulate collagen mRNA. The up-regulation was abolished by DLPC + SAMe and similarly by catalase, implicating an H_2O_2-mediated process.

To substantiate the role of H_2O_2, we assessed the capacity of leptin and menadione to generate the peroxides. It was found that H_2O_2 rose 10 times from 2.4 μM/mg

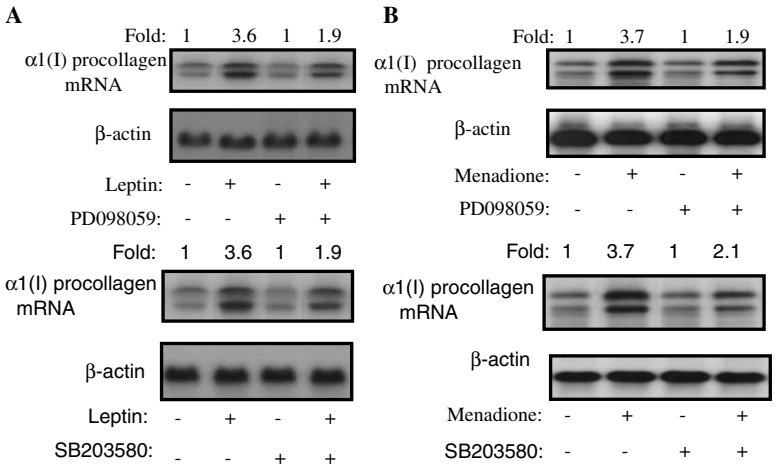

Fig. 5. ERK1/2 inhibitor PD098059 and p38 inhibitor SB203580 partially block α1(I) collagen mRNA up-regulation by leptin and menadione. LX-2 cells were treated with leptin with or without PD098059, or SB203580 (A). Similarly, cells were treated with menadione with or without PD098059, or SB203580 (B). The levels of collagen mRNA were analyzed by Northern blots 24 h after the treatment. β-Actin was used for normalization of the collagen mRNA bands. The numbers above the blots are values of three separate analyses and are expressed as fold relative to controls (no leptin and inhibitors or no menadione and inhibitors).

protein in control cells to 24 μM after 1 h of leptin treatment. This magnitude of increase was matched by menadione. Since the TD (toxicity dose)$_{50}$ for H_2O_2 in rat HSCs and in a HSC line has been reported to be 280–310 μM [11], the level of H_2O_2 (24 μM) generated in LX-2 cells by leptin or menadione is unlikely to elicit toxicity and apoptotic cell death; rather the H_2O_2 serves in the signal transduction of ERK1/2 and p38 pathways. This interpretation is consistent with the observations that H_2O_2 at a 25–50 μM concentration serves to activate the p38 pathway to up-regulate α1(I) collagen mRNA in response to TGF-β and acetaldehyde in HSCs [12–14].

Although leptin and menadione have an equivalent capacity to generate H_2O_2, the mechanism by which leptin generates H_2O_2 differs from that of menadione. We previously showed that leptin generates H_2O_2 through the leptin receptor long form which activates the Janus kinases (JAK) 1 and 2 which, in turn, stimulate H_2O_2 production [1]. While DLPC and SAMe have no inhibitory effects on the up-regulation of the receptor expression and JAK activation caused by leptin, they diminish leptin-stimulated H_2O_2 production [4]. Menadione is a quinone compound that undergoes redox cycling resulting in the formation of superoxide [5,15,16]. Enzymatic or spontaneous dismutation of superoxide yields H_2O_2 and O_2. Regardless of its sources in LX-2 cells, H_2O_2 production was attenuated by DLPC + SAMe by 70–72%, whereas it was abolished by catalase. These changes inactivated H_2O_2 signaling through the ERK1/2 and p38 pathways, resulting in inhibition of collagen mRNA expression.

Generation of H_2O_2 by leptin is coupled to a 38% decrease of cellular GSH in LX-2 cells, reflecting a consumption of GSH in response to the peroxides. It is well documented that menadione depletes cellular GSH (5, 17). Menadione possesses an electrophilic carbon center which binds cellular soft nucleophils such as reduced GSH and protein thiols [16,17]. Orrenius and co-workers [5,17] first reported that, in isolated hepatocytes, menadione (at 30–50 μM) binding to GSH produced a rapid and extensive decrease of GSH levels, thereby predisposing the cells to oxidative stress. The ability of menadione to deplete GSH in LX-2 cells was verified in the present study. We found a 43% decrease of GSH after treatment with 30 μM of menadione. Using 100 μM of menadione, Montiel-Durate [18] observed an 80% decrease of GSH accompanied by apoptosis in a rat HSC line. Importantly, the depletion of GSH, whether caused by leptin or menadione, was corrected by DLPC and SAMe combined and by catalase which blocked H_2O_2 production.

As a compensatory mechanism for GSH consumption due to H_2O_2 production, SAMe is utilized via the transsulfuration pathway to replenish the cellular GSH [19,20], which scavenges the peroxide, thereby correcting, at least partially, the leptin- or menadione-induced H_2O_2 formation in LX-2 cells. As shown and discussed in detail before [4], SAMe alone only partially (~40%) replenished GSH level after leptin treatment. A complete normalization of the oxidative stress required the synergistic actions of SAMe and DLPC. DLPC has high bioavailability and capacity for incorporation into cell membranes. The formation of phosphatidylcholines (PCs) requires SAMe for methylation. Therefore, one could anticipate that DLPC, by providing PCs, decreases SAMe utilization for PC synthesis and restores the cellular SAMe concentration with replenishment of GSH. It has also been postulated that DLPC could provide a "trap" for free radicals, thereby attenuating their adverse effects [21].

In conclusion, as antioxidants, DLPC and SAMe prevent H_2O_2 generation and restore GSH depletion caused by leptin and menadione. Blocking H_2O_2 signaling through

ERK1/2 and p38 pathways results in a complete inhibition of leptin- or menadione-induced α1(I) collagen mRNA expression. In conjunction with the prevention of TIMP-1 production, as shown before [1], the ability of DLPC and SAMe to inhibit α1(I) collagen mRNA expression provides a mechanistic basis for the prevention of hepatic fibrosis by these innocuous compounds, because enhanced TIMP-1 and collagen productions are associated with hepatic fibrogenesis and their attenuation diminishes fibrosis.

Acknowledgments

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