Regulation of Cell Growth by Redox-mediated Extracellular Proteolysis of Platelet-derived Growth Factor Receptor β*

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Redox-regulated processes are important elements in various cellular functions. Reducing agents, such as N-acetyl-L-cysteine (NAC), are known to regulate signal transduction and cell growth through their radical scavenging action. However, recent studies have shown that reactive oxygen species are not always involved in ligand-stimulated intracellular signaling. Here, we report a novel mechanism by which NAC blocks platelet-derived growth factor (PDGF)-induced signaling pathways in hepatic stellate cells, a fibrogenic player in the liver. Unlike in vascular smooth muscle cells, we found that reducing agents, including NAC, triggered extracellular proteolysis of PDGF receptor-β, leading to desensitization of hepatic stellate cells toward PDGF-BB. This effect was mediated by secreted mature cathepsin B. In addition, type II transforming growth factor-β receptor was also down-regulated. Furthermore, these events seemed to cause a dramatic improvement of rat liver fibrosis. These results indicated that redox processes impact the cell’s response to growth factors by regulating the turnover of growth factor receptors and that “redox therapy” is promising for fibrosis-related disease.

Reactive oxygen species are known to activate intracellular signal molecules (1). Extracellular signal-regulated kinase is one of the reactive oxygen species-responsive serine/threonine kinases (2). A recent study showed that hydrogen peroxide (H₂O₂) directly attacks Gαi and Gq to activate extracellular signal-regulated kinase in rat neonatal cardiomyocytes (3). In addition to the direct activation of signal molecules, H₂O₂ was also reported to contribute to the phosphorylation of mitogen-activated protein kinase in vascular smooth muscle cells (VSMC) under the stimulation of the platelet-derived growth factor (PDGF) (4). However, recent studies have forced reconsideration of this hypothesis. In HepG2 cells, PDGF receptor (PDGFR) was autophosphorylated independently of H₂O₂ production (5). In epithelial cells, interleukin-1β activated NF-κB without H₂O₂ production (6). These evidences confirmed that reactive oxygen species are not always involved in ligand-induced signaling pathways.

The hepatic stellate cell (HSC), a liver-specific pericyte, plays a central role in liver fibrogenesis (7). When undergoing activation, the HSC proliferates and generates a large amount of extracellular matrix materials including fibril-forming collagens, fibronectin, and proteoglycans, resulting in septation in chronically damaged liver. Because recent studies have confirmed that PDGF is the most potent mitogen for HSC (8), regulation of PDGF-stimulated HSC proliferation would serve as a therapeutic target for liver fibrosis. Here we show that reducing agents, including N-acetyl-L-cysteine (NAC), have the potential to disturb PDGF-dependent signal transductions and DNA synthesis in HSC. Molecular analysis revealed that NAC triggered extracellular proteolysis of PDGFR-β through the activation of a thiol-protease, cathepsin B, independently of its H₂O₂ scavenging action.

EXPERIMENTAL PROCEDURES

Materials—Collagenase and thioacetamide (TAA) were purchased from Wako Pure Chemical Co. (Osaka, Japan). Pronase E was purchased from Merck. PDGF-BB was purchased from R & D Systems (Minneapolis, MN). The polyclonal antibodies against PDGFR-α and PDGFR-β, type I transforming growth factor-β receptor (TGF-βRII), and TGF-βRII were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The monoclonal antibodies against cathepsin B and phospho-tyrosine were purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). The polyclonal antibodies against phosphoextracellular signal-regulated kinase and phospho-Akt were purchased from New England Biolabs (Beverly, MA). The monoclonal antibody against smooth muscle α-actin (α-SMA), desmin, vimentin, and fibrillar acidic protein were purchased from DAKO A/S (Glostrup, Denmark).

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Fig. 1. Effect of NAC on PDGF-BB-dependent DNA synthesis and signal transduction of primary cultured HSC. A, HSC were stimulated with 20 ng/ml PDGF-BB for 24 h after the pretreatment with NAC at the indicated dose for 24 h. DNA synthesis of HSC was measured by [3H]thymidine incorporation during the final 6 h. Results represent the means ± S.D. of three different experiments. B, analysis of PDGFR-β subunit autophosphorylation after the treatment with NAC. Confluent HSC deprived of serum for 24 h were stimulated with 20 ng/ml PDGF-BB for 10 min after the pretreatment with NAC at the indicated dose for 24 h. PDGFR-β subunits in the cell lysates were immunoprecipitated using anti-PDGFR-β antibody and then immunoblotted for phosphotyrosine using 4G10. C, Western blot analysis of phospho-p44/42 MAP kinase and total p44/42 MAP kinase. Confluent HSC deprived of serum for 24 h were stimulated with 20 ng/ml PDGF-BB for 10 min after the pretreatment with NAC at the indicated dose for 24 h. D, Western blot analysis of phospho-Akt and total Akt in PDGF-BB-stimulated HSC after the pretreatment with NAC at the indicated dose for 24 h. E, analysis of total PDGFR-β after the treatment with NAC. PDGFR-β subunits in HSC after the treatment with NAC at the indicated dose for 24 h were immunoprecipitated with anti-PDGFR-β antibody and then immunoblotted for PDGFR-β. F, measurement of 125I-PDGF-BB binding capacity to HSC. Confluent HSC were preincubated for 24 h in the presence of NAC at the indicated dose and then incubated with 1 ng/ml 125I-PDGF-BB for 2 h at 4 °C. Nonspecific binding was determined and was subtracted from the total binding to determine the specific binding. Values shown are cpm bound/10^4 cells. Data represent the means ± S.D. of three different experiments.
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Fig. 2. Regulation of PDGFR-β expression in primary cultured HSC by NAC. A, Western blot analysis of PDGFR-β in HSC after the treatment with NAC at the indicated dose for 24 h. B, Western blot analysis of PDGFR-β in HSC after the treatment with 20 mM NAC for the indicated time. C, reversibility of PDGFR-β expression after the removal of NAC. We cultured HSC in the absence of NAC for 4 days. Then we added NAC at 20 mM to the culture medium for 24 h. Afterward, we removed NAC from the culture medium during days 6–8. Then, we added NAC at 20 mM to the culture medium for 24 h and then removed NAC again. D, Western blot of PDGFR-β in HSC treated with BSO and/or NAC at the indicated dose for 24 h. E, Northern blot analysis of PDGFR-β mRNA expression in HSC treated with NAC at the indicated dose for 24 h. Equal loading of samples (20 μg) was checked by determining the expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a housekeeping gene. Ribosome RNA (28 and 18 S) was detected by staining the membrane with ethidium bromide. F, effect of NAC on PDGFR-β turnover by metabolic labeling experiments. Confluent HSC deprived of methionine for 24 h were incubated with 0.1 mCi of [35S]methionine for 24 h. Then we removed [35S]methionine from the culture medium. HSC were exposed to NAC at the indicated dose for 24 h.

Mallory Azan Staining and Morphometric Analysis of Fibrotic Area—We stained the paraffin-embedded sections (4-μm thickness) with Mallory azan, followed by morphometric analysis of fibrotic area using Mac SCOPE version 2.5 (MITANI Corp.) and Photragr-2500 for Macintosh FUJIX SH-25/M (FUJIFILM, Tokyo, Japan). Five nonoverlapping areas were evaluated in each group.

Immunohistochemistry—In immunohistochemistry, tissue sections were treated with 1% hydrogen peroxide, 0.1% proteinase K, and 1% Triton X-100. They were reacted with 1:50-diluted mouse monoclonal antibody against α-SMA overnight at 4 °C, followed by reaction with 1:200-diluted biotin-conjugated rabbit anti-mouse IgG (H+L)2, for 1 h at room temperature.

Statistical Analysis—Data presented as bar graphs are the means ± S.D. of three independent experiments except for in vivo analysis of five independent experiments. Luminograms and autoradiograms are representative of at least three experiments. Statistical analysis was performed by Student’s t test (p < 0.05 was considered significant).

RESULTS

Treatment of HSC with NAC inhibited PDGF-BB-dependent DNA synthesis in a dose-dependent manner (Fig. 1A). NAC impeded the phosphorylation of tyrosine residue of PDGFR-β and the activation of both MAP kinase and Akt dose-dependently under PDGF-BB stimulation (Fig. 1, B–D). Notably, NAC down-regulated the protein level of PDGFR-β dose-dependently (Fig. 1E). 125I-PDGF-BB binding experiments showed that pretreatment with NAC for 24 h decreased the specific binding of PDGF-BB to HSC (Fig. 1F). These results indicate that NAC blocked PDGF-dependent signaling pathways and DNA synthesis in HSC through the down-regulation of PDGFR-β protein.

NAC triggered down-regulation of PDGFR-β protein in a dose- and time-dependent manner (Fig. 2, A and B). Since removal of NAC restored PDGFR-β, this effect of NAC was not...
derived from its cytotoxic action (Fig. 2C). BSO, an inhibitor for GSH production, did not restore the down-regulation of PDGFR-β (Fig. 2D). This result indicated that GSH endogenously produced is not involved in this effect of NAC. Northern blot analysis showed that NAC had no effect on the expression of PDGFR-β mRNA (Fig. 2E). Metabolic labeling experiments revealed that NAC reduced de novo synthesized PDGFR-β (Fig. 2F). These results indicated that this effect of NAC was presumably due to accelerating degradation of PDGFR-β that had already been synthesized.

To clarify the mechanism by which NAC down-regulates PDGFR-β protein in HSC, we tested whether protease inhibitors affected NAC-induced degradation of PDGFR-β protein. EDTA and 1,10-phenanthroline are inhibitors of matrix metalloprotease; phenylmethlysulfonyl fluoride inhibits a serine protease; chymostatin inhibits chymotrypsin, papain, and cathepsin A, B, and D; leupeptin inhibits trypsin, plasmin, papain, and cathepsin B; E64 inhibits thiol proteases including cathepsin B, H, and L; and calpain; CA074 inhibits cathepsin B; and pepstatin A inhibits cathepsin D. Among these protease inhibitors, chymostatin, leupeptin, E64, CA074, and pepstatin A hampered NAC-induced down-regulation of PDGFR-β (Fig. 3A). Proteasome inhibitors such as MG115 and PSI failed to affect the degradation of PDGFR-β by NAC. These results indicate that down-regulation of PDGFR-β protein expression is mediated through its degradation by cathepsin B and/or cathepsin D.

Next, we determined whether cathepsin B or cathepsin D could degrade PDGFR-β and that NAC is required for the activation of cathepsin B.

Western blot analysis showed that reducing agents, such as L-cysteine, GSH, 2-ME, and dithiothreitol (DTT), and nonreducing agents, such as l-cystine and GSSG, and Trolox, a water-soluble vitamin E, on the expression of PDGFR-β. D, effect of membrane-permeable and impermeable thiol protease inhibitors on the effect of NAC. E, determination of the presence of cathepsin B in the culture medium of HSC and VSMC by Western blot. Confluent HSC or VSMC were deprived of serum in 100-mm dishes and then cultured for 24 h. The culture medium (1 ml) was centrifuged to remove the debris, followed by concentration into 10 μl using Microcon YM-100 (Millipore).
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Fig. 4. Effect of NAC on DNA synthesis and PDGF-BB-dependent signalings in VSMC. A, Western blot analysis of total PDGFR-β in VSMC treated with NAC at the indicated dose for 24 h. B, Western blot analysis of phosphorylated PDGFR-β in PDGF-BB-stimulated VSMC after the pretreatment with NAC at the indicated dose for 24 h. C, Western blot analysis of phospho-p44/42 MAP kinase and total p44/42 MAP kinase in PDGF-BB-stimulated VSMC after the pretreatment with NAC at the indicated dose for 24 h. D, Western blot analysis of phospho-Akt and total Akt in PDGF-BB-stimulated VSMC after the pretreatment with NAC at the indicated dose for 24 h. E, effect of NAC on PDGF-BB-stimulated DNA synthesis of VSMC. Confluent VSMC were stimulated with 20 ng/ml PDGF-BB for 24 h after pretreatment with NAC at the indicated dose for 24 h. DNA synthesis of HSC was measured by [3H]thymidine incorporation during the final 6 h. Results represent the means ± S.D. of three different experiments.

indicate that reducing agents play an essential role in the enhancement of the catalytic activity of cathepsin B, which is consistent with the general character of thiol proteases (13).

Next, we tested the site where cathepsin B degraded PDGFR-β. E64-c is a membrane-impermeable derivative of E64 (14). E64-d is a membrane-permeable derivative of E64 (15). CA074 is a membrane-impermeable derivative (16). Both E64-c and CA074 were able to restore the PDGFR-β protein similarly to E64-d (Fig. 3D). These results indicate that PDGFR-β might be degraded extracellularly by cathepsin B in the presence of NAC. In fact, mature cathepsin B as well as procathepsin B was present in the culture medium of activated HSC (Fig. 3E). This is consistent with the report by Kristensen et al. (17) showing that proteome analysis detected cathepsin B in the culture supernatant of HSC.

In sharp contrast to HSC, VSMC secreted procathepsin B, but not mature cathepsin B, into the culture medium (Fig. 3E). Here, we assumed that NAC might have no effect on the expression of PDGFR-β in VSMC. As expected, NAC failed to affect both the protein level of PDGFR-β and tyrosine phosphorylation of PDGFR-β under PDGF-BB stimulation in VSMC (Fig. 4, A and B). Accordingly, NAC had no effect on the phosphorylation of MAP kinase and Akt under PDGF-BB stimulation in a dose-dependent manner, as previously reported (Fig. 4E) (4). These results indicate that NAC may block some signal molecules except for PDGFR-β, MAP kinase, and Akt in VSMC.

Next, we examined whether the above mentioned effect of NAC was specific for PDGFR-β. NAC was also found to down-regulate the expression of TGF-βRII (Fig. 5). In contrast, NAC had no effect on the expression of other receptors, such as PDGFR-α and TGF-βRI. NAC also failed to affect the level of cytoskeletal proteins, such as α-SMA, desmin, vimentin, and glial fibrillary acidic protein (Fig. 5).

The above mentioned results indicated that NAC may suppress liver fibrosis by inhibiting HSC proliferation through inducing unresponsiveness to growth factors, such as PDGF-β and TGF-β. To test this hypothesis, we prepared two different liver fibrosis models. First, we examined whether NAC could suppress thioacetamide (TAA)-induced liver fibrosis. Mallory azan staining revealed that fibrosis was well developed in the liver of rats treated with TAA for 6 weeks (fibrotic area 8.16 ± 1.24%) (Fig. 6, A and K). NAC treatment either intraperitoneally or orally suppressed fibrosis of the liver (1.79 ± 0.51 or 2.51 ± 0.90%, p < 0.01) (Fig. 6, B, C, and K). Immunostaining of α-SMA, a marker of myofibroblast and activated HSC, showed that, while α-SMA-positive cells were numerous in and around fibrotic septum of TAA-treated livers (Fig. 6G), administration of NAC suppressed the increase of α-SMA-positive cells (Fig. 6H). This result indicated that NAC inhibited the proliferation of activated HSC also in in vivo fibrosis models. To further test whether NAC had the potential to restore the liver fibrosis that had already been developed, we started NAC treatment 6 weeks after TAA administration and excised liver tissues for histologic evaluation 3 weeks later. Liver fibrosis progressed with thick bundles of elastic fibers after 9-week administration of TAA (12.44 ± 1.74%) (Fig. 6, D and K). However, NAC treatment during the last 3 weeks either intraperitoneally or orally restored the fibrotic liver tissue to almost physiological architecture (1.79 ± 0.51 or 2.51 ± 0.90%, p < 0.01) (Fig. 6, E, F, and K).

Next, we examined the effect of NAC on the bile duct ligation model. Bile duct ligation for 2 weeks induced cholestasis, peri-ductular hepatocyte necrosis with leukocyte accumulation, and fibrosis around the portal area (3.50 ± 2.06%) (Fig. 6, I and K).
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When we administered NAC on a daily basis, the deposition of extracellular matrix was much less than that in nontreated rats (1.45 ± 1.49%, p < 0.05) (Fig. 6, J and K).

Finally, we examined whether NAC could down-regulate growth factor receptors in rat livers. Western blot analysis revealed that neither PDGFR-β protein nor TGF-βRII protein was detectable in liver tissues of rats treated with TAA and NAC (Fig. 6L). In contrast, the protein level of PDGFR-α, TGF-βRI, and α-SMA showed limited suppression by NAC administration. These results indicate that NAC has a potential to induce degradation of both PDGFR-β and TGF-βRII in vivo.

DISCUSSION

Redox regulation by intrinsic reducing suppliers, including GSH and thioredoxin, is essential to maintain intact cellular functions against oxidative stress in many mammalian cells (18). Both GSH and thioredoxin regulate cell growth and death by modulating superoxide-sensitive transcription factors, such as AP-1 and NF-κB (19). Likewise, extrinsic reducing suppliers, such as NAC, are known to regulate cell growth by increasing intracellular GSH production. NAC has been clinically utilized for acute respiratory distress syndrome, cancer, chronic bronchitis, heart disease, heavy metal poisoning, human immunodeficiency virus infection, and acetaminophen-induced liver injury (20).

Our current study provides a new insight into the pathophysiological role of redox processes. We found that the extracellular reduced condition may be an important factor for the regulation of growth factor-stimulated cellular response through the degradation of membrane receptors assisted with secreted cathepsin B. However, such a phenomenon was observed after the long term exposure of HSC to NAC. In in vitro culture, NAC at 20 mM required 24 h for inducing complete degradation of PDGFR-β. In contrast, we found that the exposure of HSC to NAC for 30 min could also inhibit PDGFR-dependent signaling without any change of PDGFR-β protein level (data not shown). Moreover, although E64 completely restored the expression of PDGFR-β at the protein level, E64-induced recovery of PDGFR-dependent DNA synthesis and signaling was limited (data not shown). These results suggest that NAC may additionally modify the PDGFR-induced signaling cascade by some unknown mechanism other than proteolytic action.

Sundaresan et al. (4) reported that, in rat VSMC, NAC blocked PDGFR-induced signaling pathways, such as MAP kinase and DNA synthesis, by scavenging H2O2. Indeed, NAC removed cytosolic H2O2 in rat HSC (data not shown). However, when measured by dichlorodihydrofluorescein diacetate (DCFH-DA), HSC at 4 days after primary culture generated detectable H2O2 without any stimulation, and PDGFR-BB failed to increase intracellular H2O2 level, while VSMC was reported to produce H2O2 only under the stimulation of PDGF (4).2 These results indicate that H2O2 may play a limited role in PDGFR-dependent signaling in HSC.

Cathepsin B belongs to a family of thiol proteases and localizes in lysosome. Cathepsin B has been reported to digest intracellularly antigens, immunoglobulin heavy chain, insulin A and B chain, glucagon, actin, myosin heavy chain, albumin, collagen, fibrinectin, proteoglycans, fibrinogen, proplasminogen activator, and trypsinogen (13). However, some studies reported that malignant cells, fibroblasts, and osteoblasts secrete cathepsin B into the extracellular space (21). These cells are reported to secrete procathepsin B but not mature cathepsin B. In contrast, we detected the presence of mature-type cathepsin B in the culture medium of HSC. Some studies showed that cathepsin D is necessary for the processing of procathepsin B to mature cathepsin B (22). Since peptatin A impeded PDGFR-β degradation (Fig. 3A), it seems that cathepsin D is also involved in the activation of cathepsin B, leading to the degradation of PDGFR-β by NAC in HSC.

Although the protective and antioxidative effect of NAC on hepatocytes should be taken into account, we showed that NAC down-regulated the expression of both PDGFR-β and TGF-βRII also in in vivo liver fibrosis models (23). TGF-β is the most potent fibrogenic factor for HSC (24). Some studies reported that inhibition of TGF-β action resulted in the suppression of [2 H. Okuyama, Y. Shimahara, N. Kawada, S. Seki, D. B. Kristensen, K. Yoshizato, N. Uyama, and Y. Yamaoka, unpublished data.]
liver fibrosis (25). Hence, we assumed that cathepsin B-mediated proteolysis of TGF-βRII might contribute to the suppression of liver fibrosis in the in vivo model. More surprisingly, NAC has the potential to reverse established liver fibrosis (Fig. 6, D–F). In this context, we assume that NAC may induce cathepsin B-mediated direct degradation of extracellular matrix. In fact, some studies showed that cathepsin B degraded extracellular matrix (26). Leto et al. (27) reported that in human serum the concentration of cathepsin B is significantly higher in patients with liver cirrhosis than in normal volunteers, indicating a clinical benefit of NAC for patients with liver fibrosis.

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