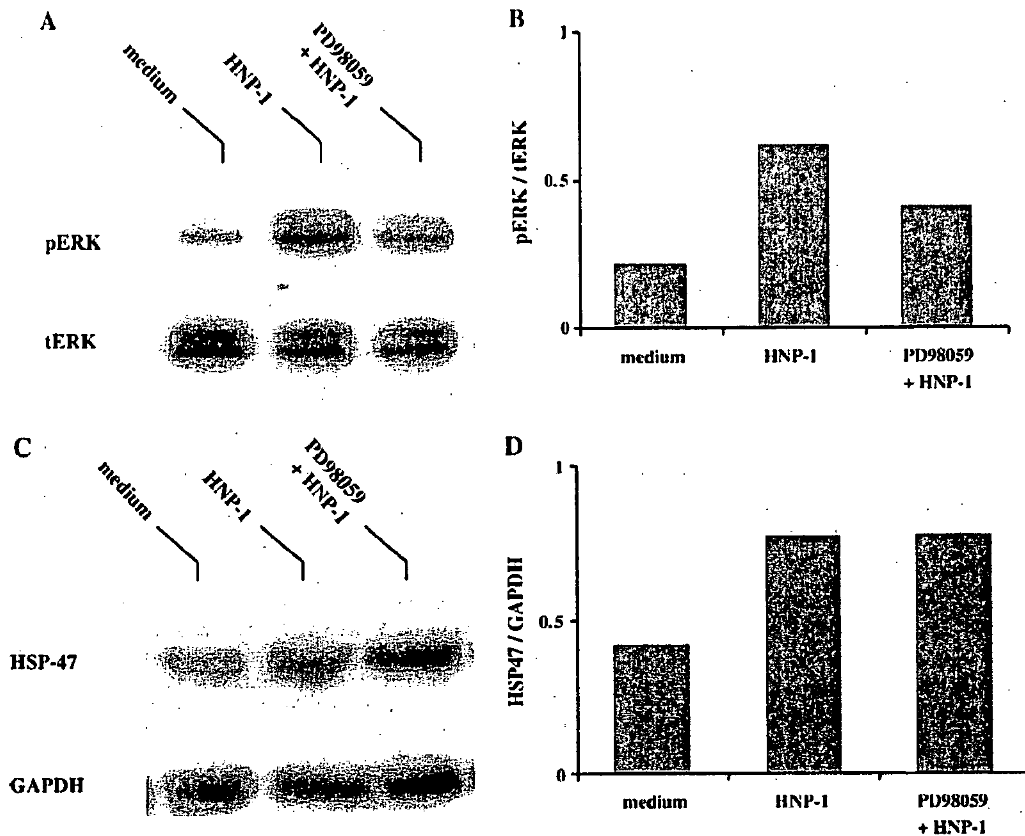

Fig. 4. (A) Activation of MAPK by HNP-1. NHLF were pre-incubated with 50 μM PD98059 for 1 h before addition of 10 μg/ml HNP-1 for 15 min. Phosphorylated ERK 1/2 (pERK) and total ERK 1/2 (tERK) were determined in cell lysates by Western blot (B). The ratio (pERK/tERK) was determined by densitometry. (C) Effect of inhibitor of MAPK signaling pathway on HSP47 protein synthesis induced by HNP-1. NHLF were pre-incubated with 50 μM PD98059 for 1 h before addition of 10 μg/ml HNP-1 for 24 h. HSP47 and GAPDH proteins were determined in cell lysates by western blot (D). The ratio (HSP-47/GAPDH) was determined by densitometry. Data represent mean of two separate experiments, each performed in duplicate.

neutrophilic inflammation is regarded as an acute inflammatory response, the persistent presence of neutrophils seems to be associated with the fibrotic response (Sibille and Marchandise., 1993). Indeed, it was reported that BAL neutrophil counts correlated with treatment response (Turner-Warwick and Haslam, 1987), morphologic extent (Wells et al., 1998), rapidly progressive (Suga et al., 2000) and untreated active disease in IPF (Crystal et al., 1984). Furthermore, an *in vitro* study demonstrated that co-culture of fibroblasts and neutrophils significantly augmented fibroblast-mediated contraction of collagen gels (Skold et al., 1999), suggesting that the interaction between neutrophils and fibroblasts is closely associated with collagen deposition. However, the exact mechanism for this remains unclear.

The roles of α-defensins in inflammatory lung diseases are currently thought to be not only antimicrobial, but also modulation of inflammatory and immune response and wound repair (van Wetering et al., 1999). Murphy et al. showed that HNP-1 induces a dose-dependent increase in DNA synthesis in mouse lens epithelial cells and NIH 3T3 fibroblasts (Murphy et al., 1993). Oono et al. also demonstrated that synthetic HNP-1 enhanced the expression of pro-α1(I) collagen in cultured human dermal fibroblasts (Oono et al., 2002). These results and our present data indicate that α-defensins could promote directly the synthesis of fibrogenic molecules in fibroblasts. In our study, HNP-1 also induced HSP47 mRNA expression and its protein production in human lung fibroblasts. Overexpression of HSP47 has been described in experimental animal models of fibrosis, including murine bleomycin-induced pulmonary fibrosis (Ishii et al., 2003; Kakugawa et al., 2004), rat peritoneal sclerosis (Nishino et al., 2003) and carbon tetrachloride-induced rat liver cirrhosis (Masuda et al., 1994) and in patients with IPF (Kakugawa et al., 2005), suggesting the importance of HSP47 in collagen synthesis in various fibrotic disorders. Thus, the fibrotic effects of HNP-1 may be mediated not only through collagen production but also through HSP 47 expression, which may lead to excessive collagen synthesis. In this context, previous reports showed a strong positive staining for HNP-1 as well as HSP47 and type 1 procollagen in areas of active fibrosis (Kakugawa et al., 2005; Mukae et al., 2002), suggesting that α-defensins released by neutrophils in the alveolar septa could directly induce excessive deposition of extracellular matrix proteins regulating HSP47 and collagen-1 in patients with IPF.

HNP-1 concentrations used in this study were higher than those in BAL fluid of patients with IPF (Mukae et al., 2002). In

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C-Mannosylated peptides derived from the thrombospondin type 1 repeat enhance lipopolysaccharide-induced signaling in macrophage-like RAW264.7 cells

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C-Mannosylation is a unique type of glycosylation occuring at the first Trp (W) in the WXXW motif, which is found in the thrombospondin type 1 repeat (TSR) of proteins. However, the biological function of C-mannosylation is not fully understood. In this study, we investigated the effect of C-mannosylated TSR-derived peptides on lipopolysaccharide (LPS)-induced signaling in macrophage-like RAW264.7 cells. The cells were stimulated with LPS in the presence or absence of chemically synthesized peptides with or without C-mannose (e.g., (C-Man)-Trp-Ser-Pro-Trp [C-Man-WSPW], C-Man-W, WSPW, etc.), then the effects of the peptides on cellular viability and signaling were examined. We found a cytotoxic effect in the cells treated with LPS and C-Man-WSPW, but not in the cells solely treated with LPS or C-Man-WSPW. We also found that production of tumor necrosis factor-α (TNF-α) was upregulated more in response to LPS plus C-Man-WSPW, than in response to LPS plus WSPW or LPS alone. Among the LPS-induced signaling pathways that induce production of TNF-α, the activation of c-Jun N-terminal kinase (JNK) was greatly enhanced by LPS and C-Man-WSPW, and the production of TNF-α was suppressed by an inhibitor for JNK. Together, these results demonstrate a novel function of the C-mannosylated TSR-derived peptide motif, to promote LPS-induced JNK signaling, and this leads to an enhancement of cytotoxicity via the upregulation of TNF-α production.

Keywords: C-mannosylation/lipopolysaccharide/macrophage/thrombospondin

Introduction

C-Mannosylation is unique in that an α-mannose is directly bound to the indole C2 carbon atom of a Trp (W) residue through a C-C bond to produce C-mannosyl Trp (C-Man-W) (Hofsteenge et al. 1994). C-Mannosylation occurs at the first Trp in the consensus amino acid sequence *Trp*-X-X-Trp (WXXW) in proteins (Furmanek and Hofsteenge 2000). The WXXW motif is found in the thrombospondin type 1 repeat (TSR), which is predicted to be a functional peptide module in various integral proteins such as thrombospondin-1 (TSP-1) (Tucker 2004). The motif is known to bind with heparin, heparan sulfate proteoglycans, and collagen, suggesting a functional significance in cell–cell interaction and/or cellular signaling. There are a number of examples of C-mannosylated proteins including ribonuclease 2 (Hofsteenge et al. 1994), Interleukin-12 (Doucey et al. 1999), complements (C6, C7, C8a, C8b, and C9) (Hofsteenge et al. 1999), properdin (Hartmann and Hofsteenge 2000), thrombospondin (Hofsteenge et al. 2001; De Peredo et al. 2002), F-spondin (Furmanek and Hofsteenge 2000), the erythropoietin receptor (Furmanek et al. 2003), mucins (MUC5AC and MUC5B) (Perez-Vilar et al. 2004). C-Mannosylation is thought to be carried out by a specific unidentified mannosyltransferase located in the microsomes. This suggests that C-mannosylation is involved in conventional glycosylation through the secretory pathway (Doucey et al. 1998).

Glycosylation has been revealed to have functional relevance to various cellular events including cell development, growth, differentiation, and death (Varki 1993; Dennis et al. 1999; Haltiwanger and Lowe 2004). However, the biological significance of C-mannosylation has yet to be fully investigated because a suitable methodology has not been established. In general, it is difficult to control the formation of glycoforms in cultured cells, which hampers progress in glycobiology and is a major obstacle to research into glycoprotein functions. One approach to conducting research in glycobiology is to generate well-defined synthetic glycopeptides or small glycoproteins using chemical or chemoenzymatic synthesis (Buskas et al. 2006). Since the discovery of C-mannosylated glycoproteins, several ways to obtain C^2-α-D-C-Man-L-Trp building blocks have been reported (Manabe and Ito 1999; Nishikawa et al. 2001). Furthermore, Manabe et al. (2003) reported a route that differs from conventional approaches to C-glycosylation (Beau and Gallagher 1997). Recently, using a synthetic C-Man-W, we prepared a specific antibody against C-Man-W, then found that C-mannosylated proteins were expressed in mouse macrophage-like RAW264.7 cells, and the expression was upregulated under hyperglycemic conditions (Ihara et al. 2005).

To know whether C-mannosylation plays a functional role in macrophages, we focused on lipopolysaccharide (LPS)-induced signaling in macrophages, and investigated the effect of chemically synthesized C-mannosylated TSR-derived peptides (e.g., (C-Man)-Trp-Ser-Pro-Trp [C-Man-WSPW] [Figure 1]) on the signaling pathway in RAW264.7 cells. Here we report that C-mannosylated TSR-derived peptides modulate lipopolysaccharide (LPS)-induced signaling including the pathways involving

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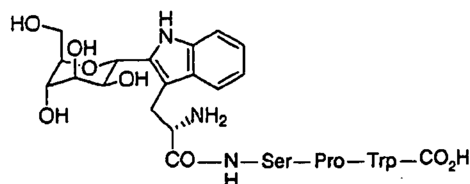

Fig. 1. The structure of C-mannosyl tryptophan in the TSR-derived peptide.

mitogen activating protein kinases (MAPKs) such as c-Jun N-terminal kinase (JNK), and enhance the cytotoxicity of LPS.

Results

C-Mannosylated TSR-derived peptides enhance LPS-induced cell damage in RAW264.7 cells

C-Mannosylated TSR-derived peptides and derivatives were chemically synthesized as described in Materials and methods. Cells were cultured for 48 h with different concentrations of LPS in the presence or absence of C-Man-WSPW or WSPW (10 μM). The WSPW peptides, in which the first W has the potential to be C-mannosylated, are derived from TSR2 of human TSP-1, and the first W corresponds to Trp423 in the amino acid sequence (Lawler and Hynes 1986). Cell viability was examined using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), as described in the methods (Figure 2A). Cell viability was little influenced by LPS between 0.1 and 5 μg/mL. In the presence of C-Man-WSPW (10 μM), viability was apparently reduced by LPS, especially at 1 μg/mL. However, no enhancement of LPS-dependent cell damage was observed in the presence of WSPW (10 μM). To further investigate the effect of C-Man-WSPW on LPS-induced cell damage, damaged cells were examined microscopically after staining with SYTOX Green, a noncell-permeable dye used to stain the nucleus (Figure 2B). Cells were cultured for 48 h with LPS (1 μg/mL) in the presence or absence of C-Man-WSPW or WSPW (10 μM). After the treatment with LPS, there was a slight enlargement of the cell body with or without C-Man-WSPW or WSPW. The cells stained with SYTOX Green were slightly observed in the cases with LPS plus WSPW or LPS alone. However, SYTOX Green-stained nuclei were observed more in the cells treated with LPS plus C-Man-WSPW, indicating that the plasma membrane was damaged in these cells. Together, these results suggest that C-Man-WSPW specifically induces damage in the cells treated with LPS.

Next, to see the dose-responsive effect of C-Man-WSPW on LPS-induced cell damage, cells were cultured for 48 h with LPS (1 μg/mL) in the presence of various concentrations of C-Man-WSPW (0.5–10 μM). Cell viability was examined as described above. The results showed that C-Man-WSPW enhanced the LPS-induced cell damage in a dose-dependent manner (Figure 2C). To further investigate the structural requirement of C-mannosylated peptides for enhancement of the damage due to LPS, cells were cultured for 48 h with or without LPS (1 μg/mL) in the presence or absence of mannose, C-Man-W, C-mannosylated peptides with different structures (e.g., C-Man-WS, C-Man-WSP, C-Man-WSPW, C-Man-WSPWS, C-Man-WSG, and C-Man-WSK) or WSPW (10 μM), then cell viability was examined. As shown in Figure 2D, C-mannosylated peptides apparently enhanced the LPS-induced cell damage, especially in the cases of C-Man-WSP, C-Man-WSPW, and C-Man-WSPWS. C-Man-WSG and C-Man-WSK also moderately enhanced LPS-induced cell damage. On the other hand, C-Man-WS, C-Man-W, and mannose had a minor effect compared with other C-mannosylated peptides, and nonglycosylated WSPW had little effect as well. Furthermore, without LPS, cell viability was not reduced by mannose, C-Man-W, C-Man-WSPW, or WSPW (10 μM). These results indicate that C-mannosylated peptides, which contain tripeptides such as WSP, effectively enhance LPS-induced damage in the cells.

Effect of C-mannosylated TSR-derived peptides on the binding of LPS to the cells

To investigate whether C-Man-WSPW influences the binding of LPS to RAW264.7 cells, the cells were incubated at 37°C for 20 min with FITC-conjugated LPS (LPS-FITC) (1 μg/mL) in the presence or absence of WSPW or C-Man-WSPW (10 μM) as described in the methods. After a wash with PBS, the binding of LPS-FITC to the cells was analyzed by flow cytometry (Figure 3A). The results showed that LPS-FITC was similarly bound to the cells in the presence of C-Man-WSPW or WSPW, indicating that C-Man-WSPW or WSPW did not directly influence the binding of LPS to the cells. Next, to determine the amount of WSPW or C-Man-WSPW bound to RAW264.7 cells, the cells were incubated with biotin, biotin-labeled WSPWC (WSPWC-biotin) or C-Man-WSPWC (C-Man-WSPWC-biotin) (10 μM), and then the molecules bound to the cells were detected by flow cytometry after incubation with FITC-conjugated avidin (Figure 3B). The results showed that C-Man-WSPWC-biotin was bound to the cells, although the binding was weak. On the other hand, biotin and WSPWC-biotin showed less of a background signal. To further examine the interaction between C-mannosylated peptides and target proteins in the cell, the cells were incubated with biotin, WSPWC-biotin, or C-Man-WSPWC-biotin (10 μM), and then the proteins bound to the biotin conjugates were detected, as described in the methods, by blot analysis using peroxidase-conjugated avidin after treatment with or without dithiobis[succinimidylpropionate] (DSP), a membrane-permeable crosslinker. As shown in Figure 3C, various proteins were bound to C-Man-WSPWC-biotin to a greater extent than those to WSPWC-biotin, under the conditions with DSP (arrows). The binding was not apparent under the conditions without DSP, although some non-specific binding of peroxidase-conjugated avidin to proteins was detected (arrow heads). These results suggest a specific but weak interaction between C-Man-WSPWC-biotin and the proteins.

Taken together, these results indicate that C-Man-WSPWC was predominantly bound to some target proteins of RAW264.7 cells, although it did not directly influence the binding of LPS to the cells.

C-Mannosylated TSR-derived peptides enhance LPS-induced production of TNF-α in the cells

LPS is cytotoxic due to the TNF-α or nitric oxide produced by the macrophages it stimulates (Guha and Mackman 2001). Thus, we focused on the effect of C-mannosylated TSR-derived peptides on the LPS-induced production of TNF-α and cell signaling. To investigate whether the C-mannosylated peptides influence the LPS-induced production of TNF-α, RAW264.7

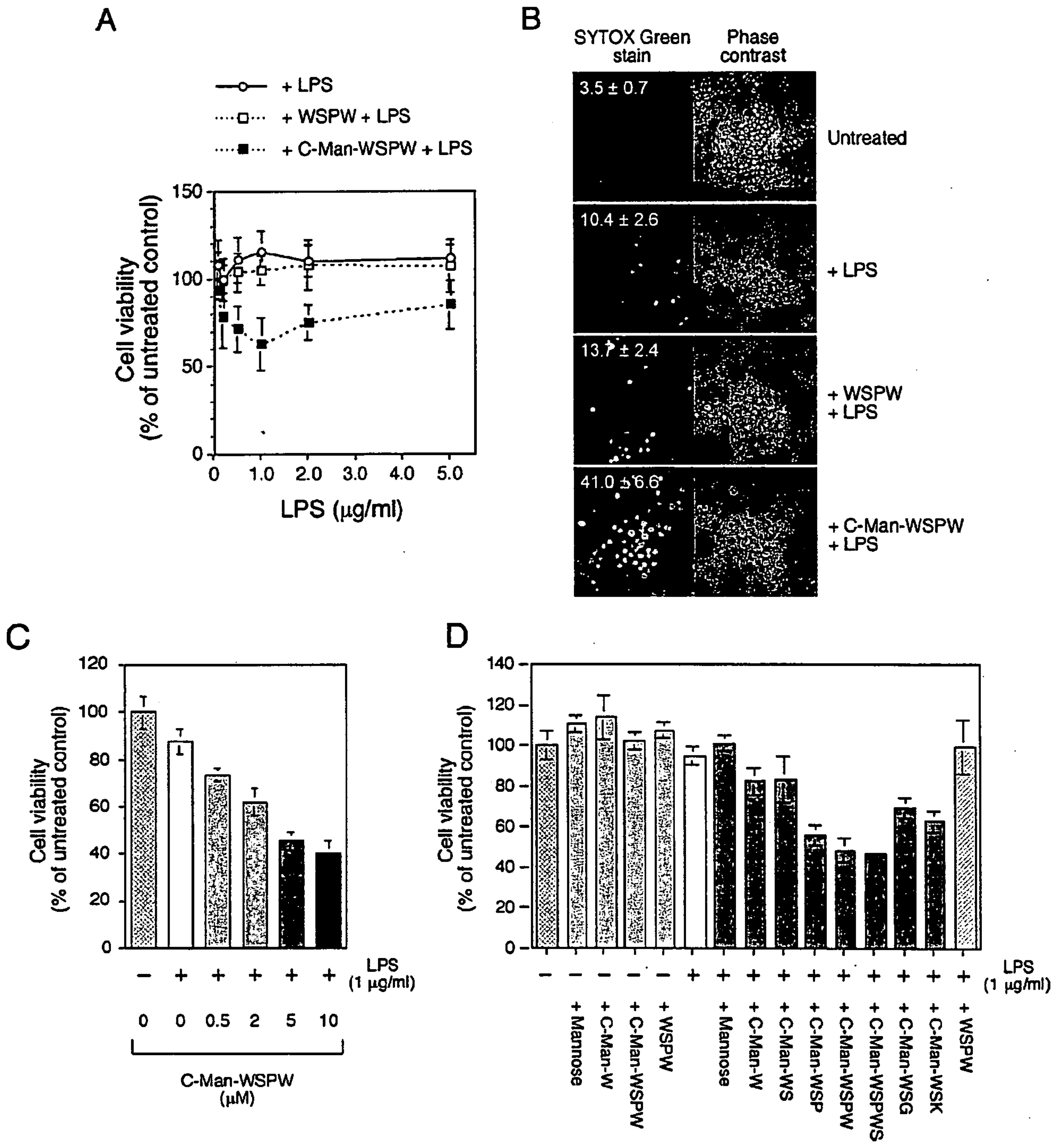

Fig. 2. C-Mannosylated TSR-derived peptides enhance the cytotoxicity of LPS in RAW264.7 cells. (**A**) RAW264.7 cells were cultured for 48 h in medium containing one of the several different concentrations of LPS in the presence or absence of C-Man-WSPW or WSPW (10 μM). Cell viability was examined by MTT assay, as described in Materials and methods. Each value represents the mean ± SD of four independent experiments. (**B**) Cells were treated as in A, then stained with SYTOX Green (1 μM) for 10 min. After fixation, signals were visualized by fluorescence microscopy with excitation at 485 nm and emission at 520 nm. Over 200 cells were counted to determine the proportion of SYTOX Green-positive cells (%). The values represent the means ± SD of three independent experiments. (**C**) Cells were cultured for 48 h with LPS (1 μg/mL) in the presence or absence of various concentrations of C-Man-WSPW (0.5–10 μM). Cell viability was examined by MTT assay, as described. (**D**) Cells were cultured for 48 h with or without LPS (1 μg/mL) in the presence or absence of mannose, C-Man-W, C-mannosylated peptides with different structures (C-Man-WS, C-Man-WSP, C-Man-WSPW, C-Man-WSPWS, C-Man-WSG, and C-Man-WSK), or WSPW (10 μM), and then cell viability was examined by MTT assay. Each value represents the mean ± SD of three to five independent experiments.

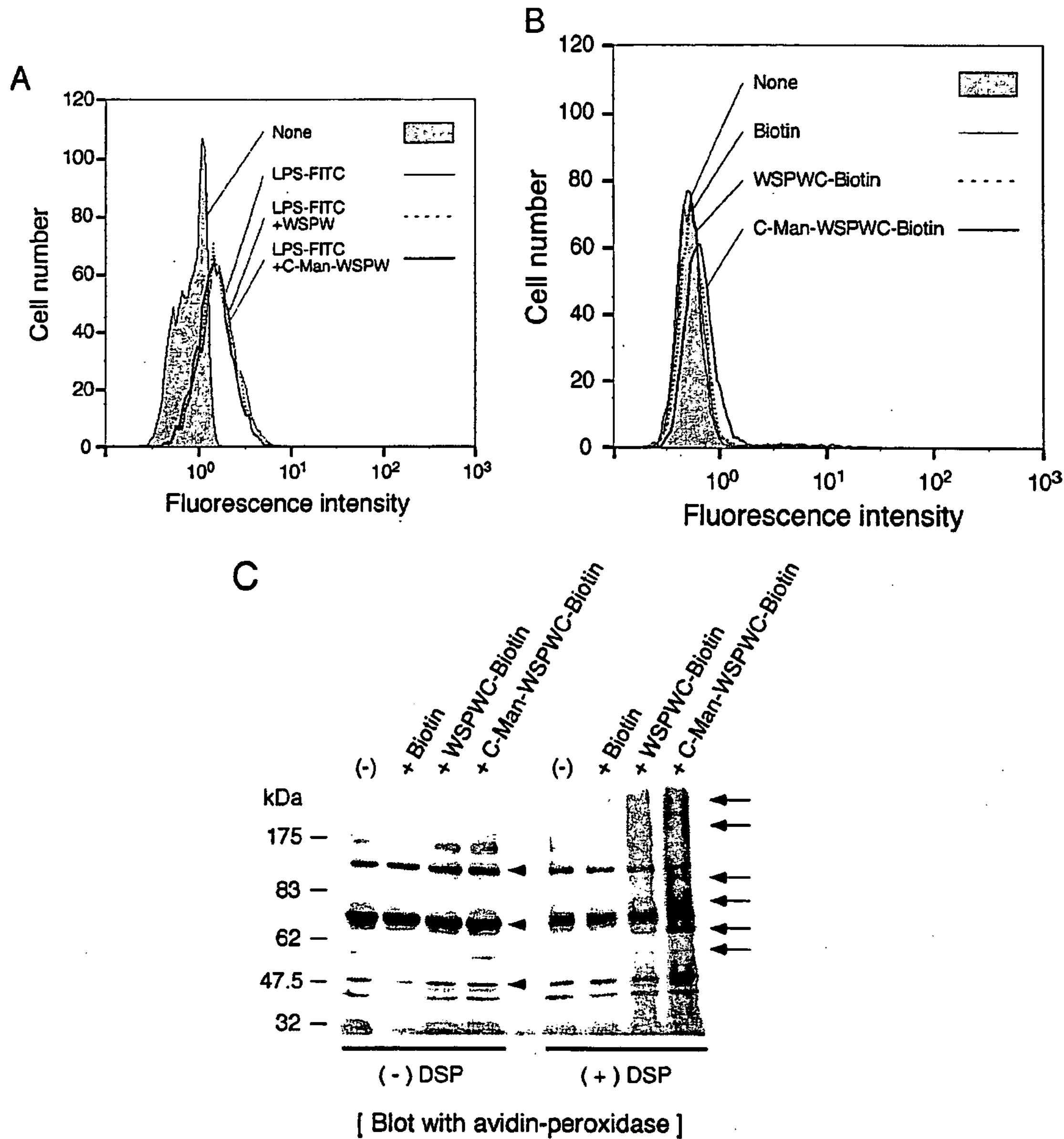

Fig. 3. Effect of C-Man-WSPW or WSPW on the binding of LPS to RAW264.7 cells. (A) Cells were incubated at 37°C for 20 min in medium containing LPS-FITC (1 μg/mL) in the presence or absence of C-Man-WSPW or WSPW (10 μM). After a wash with PBS, the binding of LPS-FITC to the cells was analyzed by flow cytometry, as described in Materials and methods. (B) Cells were incubated at 37°C for 20 min in PBS with biotin, WSPWC-biotin, or C-Man-WSPWC-biotin (10 μM). After a wash with PBS, the cells were blocked with 3% BSA in PBS, incubated with FITC-conjugated avidin for 15 min, and washed with PBS containing 1% BSA. After another wash, the binding of biotin conjugates to cells was analyzed by flow cytometry. (C) Cells were incubated with the biotin conjugates as described in B, and treated for 10 min with or without 1 mM DSP. After quenching with 1M Tris–HCl (pH 7.2), cells were washed with PBS, and subsequently harvested. The cells were lysed and protein samples were electrophoresed on 7.5% SDS-polyacrylamide gels under nonreducing conditions and then transferred to a nitrocellulose membrane. The membrane was blocked with 5% skim milk in TBS and then incubated at 4°C for 10 min with the peroxidase-conjugated avidin in TBS containing 0.1% Tween 20. After a wash with TBS containing 0.1% Tween 20, the blots were developed using an ECL chemiluminescence detection kit.

cells were treated with LPS (1 μg/mL) in the presence or absence of C-Man-WSPW or WSPW (10 μM), then the amount of TNF-α secreted from the cells was estimated by enzyme-linked immunoabsorbent assay (ELISA). As shown in Figure 4A, secretion of TNF-α increased in the culture medium following the treatments. In the case of LPS plus C-Man-WSPW, the secretion was especially enhanced at 1 and 2 h after the treatment, compared with the conditions with LPS plus WSPW or LPS alone.

The transcriptional expression of TNF-α was also examined by reverse transcription-polymerase chain reaction (RT-PCR) in the cells treated with LPS in the presence or absence of C-Man-WSPW or WSPW. The cells were treated for 1 and 2 h with LPS (1 μg/mL) in the presence or absence of C-Man-WSPW or WSPW (10 μM), then total RNA was extracted and subjected to RT-PCR. As shown in Figure 4B, the transcriptional level of TNF-α was enhanced more with LPS plus C-Man-WSPW than

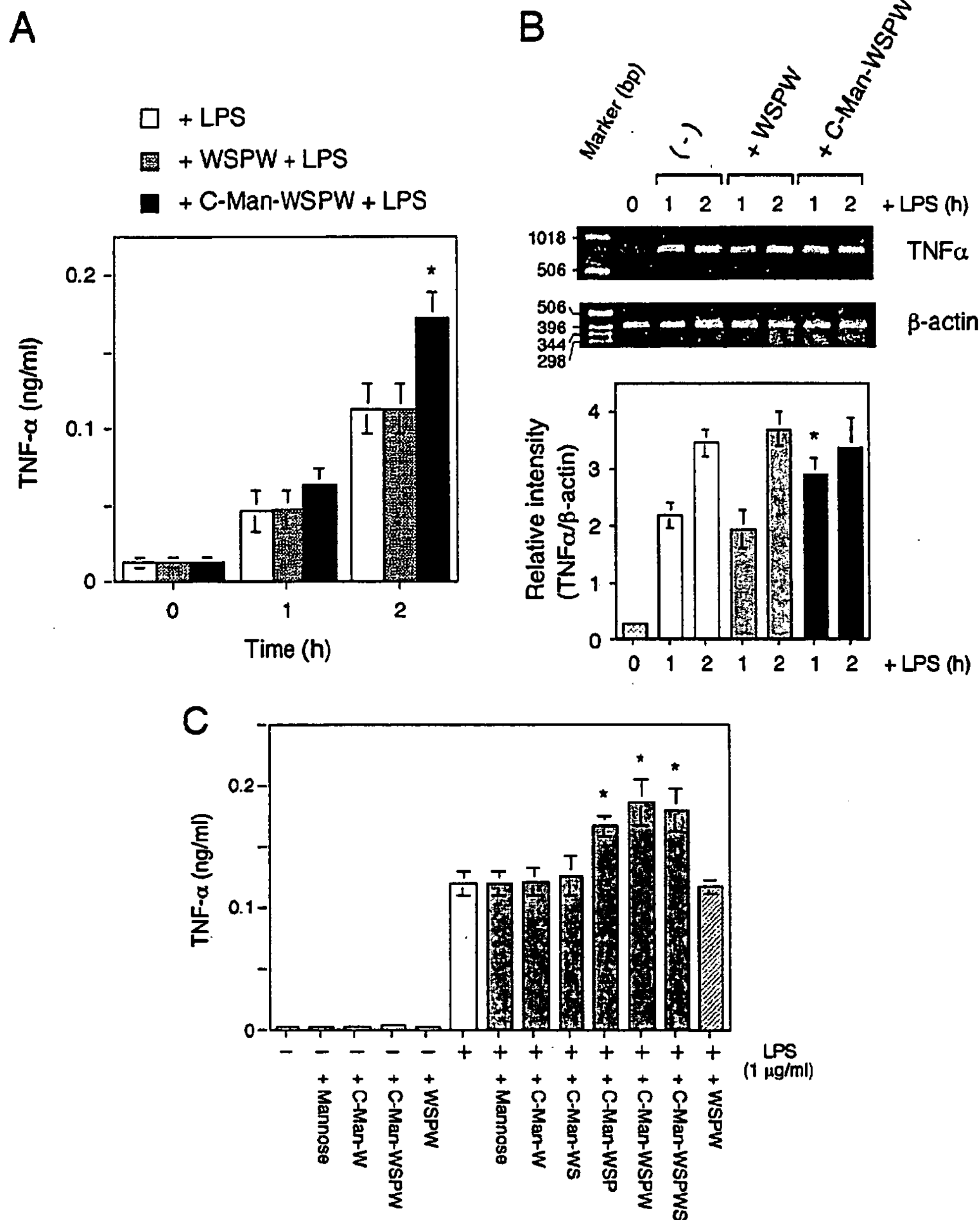

Fig. 4. C-Mannosylated TSR-derived peptides enhance the LPS-induced production of TNF-α in RAW264.7 cells. (A) Cells were plated in 96-well plates (10,000 cells/well), and cultured overnight. After being washed, the cells were stimulated for the periods indicated with LPS (1 μg/mL) in the presence or absence of C-Man-WSPW or WSPW (10 μM). After the incubation, the culture medium was collected, and the concentration of TNF-α in the sample was measured using a mouse TNF-α ELISA kit as described in Materials and methods. Each value represents the mean ± SD of four independent experiments. $^*P < 0.05$ vs. value for the cells treated with LPS or WSPW plus LPS. (B) Cells were stimulated for the periods indicated with LPS (1 μg/mL) in the presence or absence of C-Man-WSPW or WSPW (10 μM). Total RNA was prepared from the cells, and a RT-PCR analysis was performed to evaluate the transcriptional expression of TNF-α. The expression of β-actin was also shown as a control. The band intensity was estimated densitometrically, and the relative value of TNF-α to β-actin represents the mean ± SE of three independent experiments ($^*P < 0.05$ vs. value at same time point (1 h) for the cells treated with LPS or WSPW plus LPS). (C) Cells were cultured for 2 h with or without LPS (1 μg/mL) in the presence or absence of mannose, C-Man-W, C-mannosylated peptides with different structures (C-Man-WS, C-Man-WSP, C-Man-WSPW, and C-Man-WSPWS), or WSPW (10 μM), and then the TNF-α concentration in the medium was evaluated using a mouse TNF-α ELISA kit. Each value represents the mean ± SD of three independent experiments ($^*P < 0.05$ vs. value for the cells treated with LPS alone).

with LPS plus WSPW or LPS alone. Next, to investigate the structural requirement of C-mannosylated peptides for the enhancement of the LPS-induced production of TNF-α, cells were cultured for 2 h with LPS (1 μg/mL) in the presence or absence of mannose, C-Man-W, *C*-mannosylated peptides with different structures (e.g., C-Man-WS, C-Man-WSP, C-Man-WSPW, and C-Man-WSPWS), or WSPW (10 μM), then the amount of TNF-α secreted was measured by ELISA as described. As shown in Figure 4C, C-mannosylated peptides, such as C-Man-WSP, C-Man-WSPW, and C-Man-WSPWS, apparently enhanced the

LPS-induced production of TNF-α, compared with LPS alone. C-Man-WSG and C-Man-WSK also similarly enhanced the LPS-induced production of TNF-α (data not shown). On the other hand, mannose, C-Man-W, and C-Man-WS had a minor effect compared with other C-mannosylated peptides, and nonglycosylated WSPW had very little effect. Furthermore, without LPS, the production of TNF-α was not triggered by mannose, C-Man-W, C-Man-WSPW or WSPW (10 μM). These results indicate that C-mannosylated peptides, which contain tripeptides such as WSP, effectively enhance the LPS-induced production of TNF-α in RAW264.7 cells. Collectively, they indicate that LPS-induced expression of TNF-α is enhanced with C-mannosylated peptides such as C-Man-WSPW.

C-Mannosylated TSR-derived peptides enhance LPS-induced activation of JNK in the cells

LPS induces a variety of cellular signaling events, including the activation of NF-κB and activation of the members of the MAPK family (Guha and Mackman 2001). The LPS signaling cascade leading to the production of TNF-α is controlled at the levels of both transcription of the TNF-α gene and translation of the mRNA. Transcriptional control of the gene is mediated primarily through the NF-κB signaling pathway (Shakhov et al. 1990), and translation of the mRNA is regulated through the JNK pathway (Swantek et al. 1997). To investigate the effect of C-Man-WSPW on NF-κB signaling with LPS, the transcriptional activity of NF-κB was examined by conducting a luciferase reporter assay for NF-κB as described in the methods. After transfection with the lufciferase vector for NF-κB, the cells were treated with LPS (1 μg/mL) in the presence or absence of WSPW or C-Man-WSPW (10 μM), and reporter activity was measured (Figure 5A). The results showed that LPS-induced activation of NF-κB was not influenced by C-Man-WSPW or WSPW. To further investigate the NF-κB signaling with LPS, degradation of inhibitor of κBα (IκBα) was also examined by immunoblot analysis using specific antibodies, because degradation of IκBα occurred during the activation of NF-κB (Guha and Mackman 2001). The results showed that there was no difference in the LPS-induced degradation of IκBα in the cells treated with or without C-Man-WSPW or WSPW (Figure 5B).

To investigate how C-Man-WSPW enhances the LPS-induced production of TNF-α, the activation status of several MAPKs was examined in the cells treated with LPS (1 μg/mL) in the presence or absence of C-Man-WSPW or WSPW (10 μM). The cells were treated with the reagents for given periods, then the phosphorylation status of MAPKs, such as extracellular signal-regulated kinase (ERK) 1/2, p38-MAPK, and JNK, was examined by immunoblot analysis using specific antibodies recognizing the phosphorylated form of each kinase. As shown in Figure 6A, B, and C, phosphorylation was induced in all tested MAPKs by the treatment with LPS, indicating that LPS upregulated MAPK signaling, and was consistent with previous findings related to LPS-induced MAPK signaling (Guha and Mackman 2001). The results also showed that the LPS-induced phosphorylation of MAPKs was little influenced by WSPW peptides. On the other hand, the LPS-induced phosphorylation was apparently enhanced by C-Man-WSPW especially

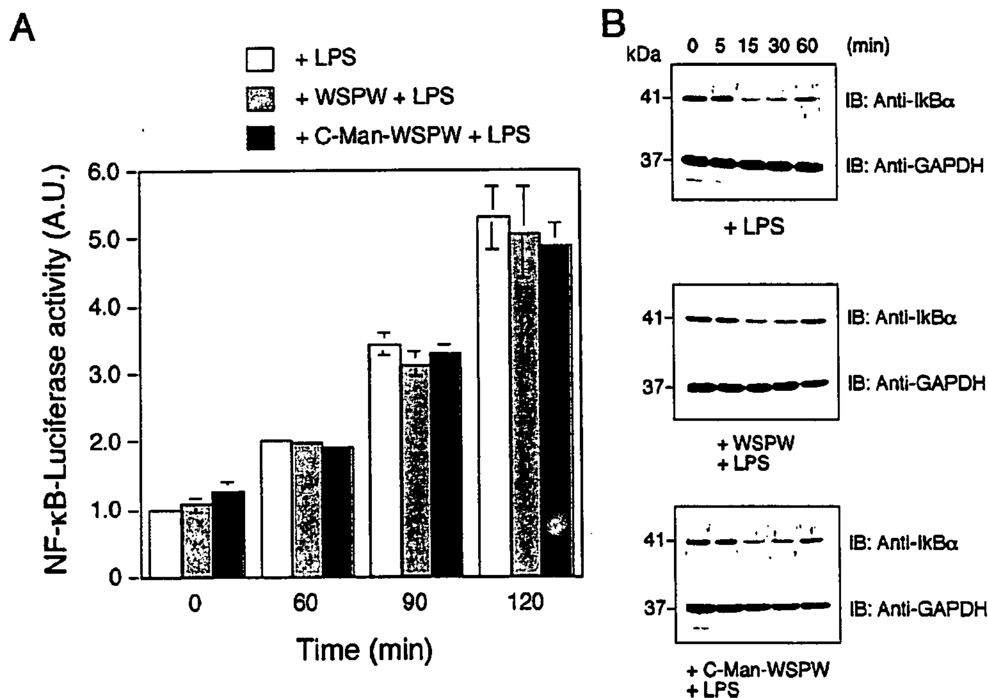

Fig. 5. Effect of C-Man-WSPW or WSPW on the NF-κB signaling in RAW264.7 cells treated with LPS. (A) After 24 h of transfection with the lufciferase vector for NF-κB, the cells were incubated at 37°C for the periods indicated in medium containing LPS (1 μg/mL) in the presence or absence of C-Man-WSPW or WSPW (10 μM), and the reporter activity was measured as described in the methods. (B) Cells were incubated at 37°C for the periods indicated in medium containing LPS (1 μg/mL) in the presence or absence of C-Man-WSPW or WSPW (10 μM). Degradation of IκBα was examined with samples of cell lysate by immunoblot analysis using specific antibodies as described in Materials and methods.

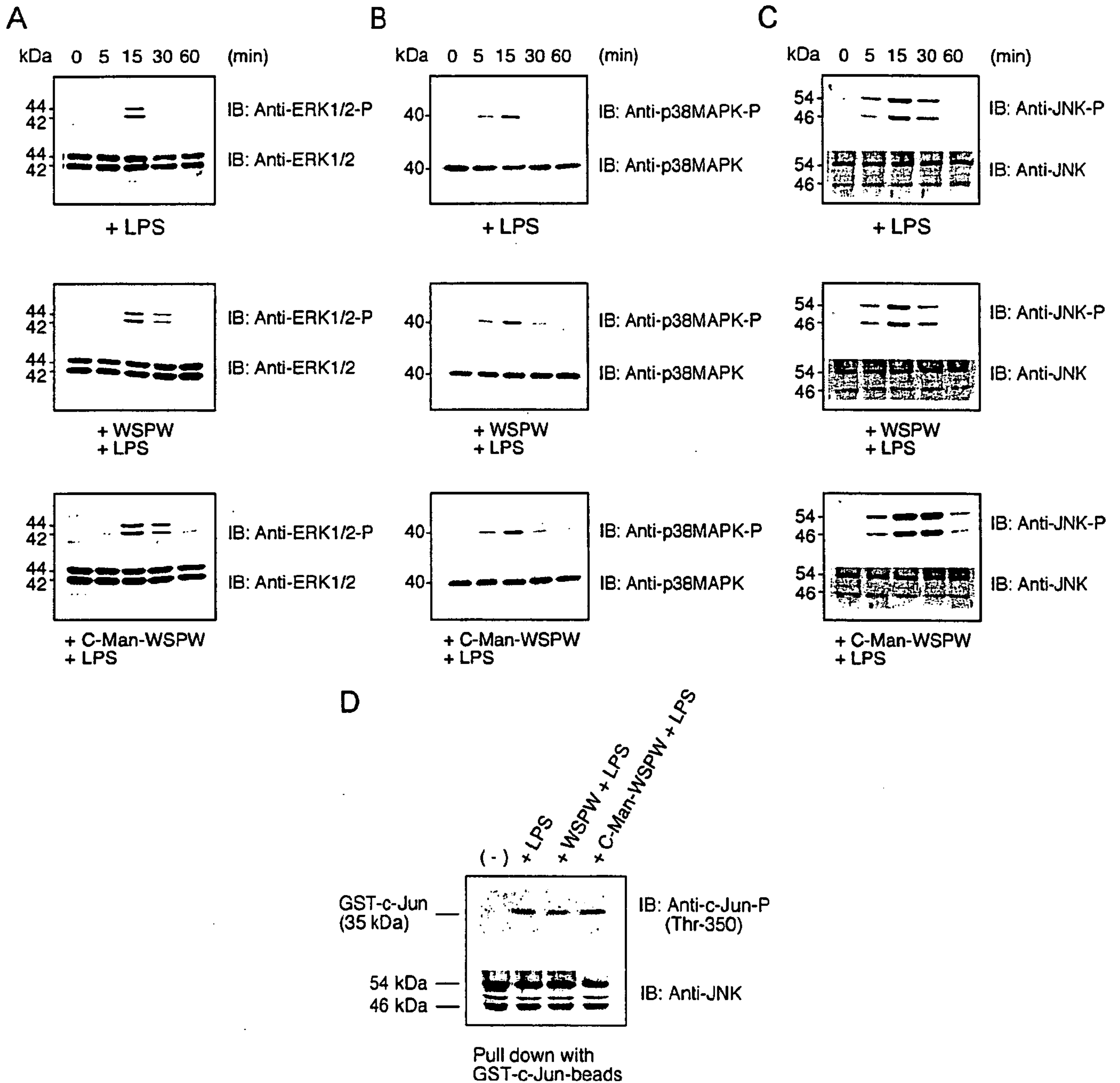

Fig. 6. Effect of C-Man-WSPW or WSPW on the MAPK signaling in RAW264.7 cells treated with LPS. A, B, C, and cells were treated with LPS, LPS plus WSPW, or LPS plus C-Man-WSPW, as described in Figure 5B. The phosphorylation status of MAPKs, such as ERK1/2 (Thr-202/Tyr-204) (A), p38-MAPK (Thr-180/Tyr-182) (B), and JNK (Thr-183/Tyr-185) (C), was examined with samples of cell lysate by immunoblot analysis using specific antibodies as described in Materials and methods. (D) The activity of JNK was examined, as described in the methods using GST-c-Jun as a substrate in the samples prepared from cells treated at 37°C for 15 min with LPS (1 μg/mL) in the presence or absence of C-Man-WSPW or WSPW (10 μM).

in the case of JNK, although slight enhancement was observed in the cases of ERK1/2 and p38-MAPK. The phosphorylation of MAPKs was not induced in the cells solely treated with WSPW or C-Man-WSPW (data not shown), indicating that LPS was required to upregulate all of the MAPK signaling. To confirm the enhanced activation of JNK in the cells treated with LPS plus C-Man-WSPW, the activity of JNK was examined using GST-c-Jun fusion protein as a substrate in samples prepared from cells treated for 15 min with LPS (1 μg/mL) in the presence or absence of C-Man-WSPW or WSPW (10 μM). As shown in Figure 6D, phosphorylation of GST-c-Jun was upregulated more in the cells treated with LPS plus C-Man-WSPW, than those treated with LPS plus WSPW or LPS alone. This indicates that JNK activity is specifically enhanced by LPS plus C-Man-WSPW. Collectively, these results indicate that LPS-induced JNK signaling was upregulated by C-Man-WSPW, but not by WSPW,

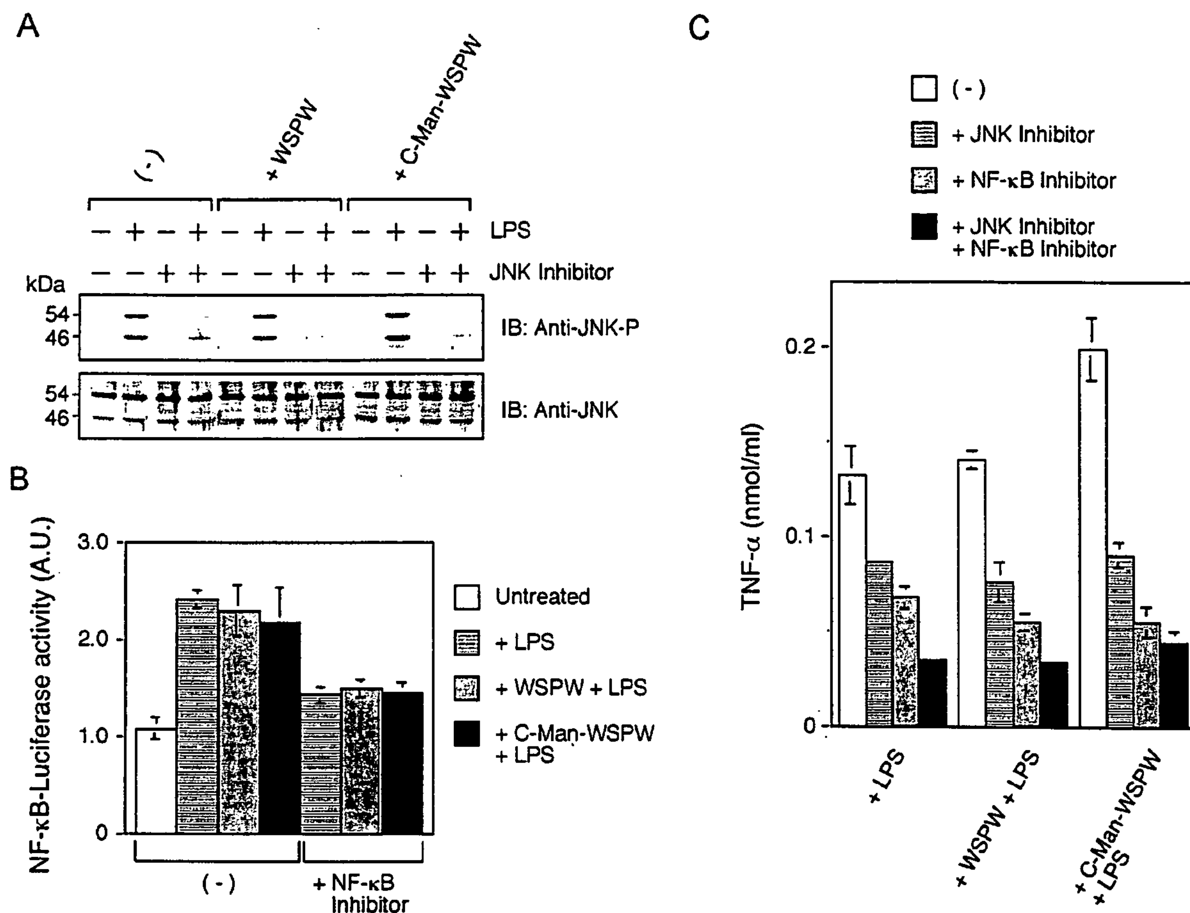

Fig. 7. JNK and NF-κB contribute to the LPS-induced production of TNF-α in RAW264.7 cells in the presence or absence of C-Man-WSPW or WSPW. (A) Cells were pretreated at 37°C for 10 min with the JNK inhibitor (SP600125, 10 μM), then treated for 15 min with LPS (1 μg/mL) in the presence or absence of C-Man-WSPW or WSPW (10 μM). The activation status of JNK was examined in the cell lysates by immunoblot analysis using the antiphospho-JNK (Thr183/Tyr-185) antibody as described in Materials and methods. (B) After 24 h of transfection with the luciferase vector for NF-κB, the cells were pretreated at 37°C for 10 min with a NF-κB activation inhibitor (10 μM), then treated for 90 min with LPS (1 μg/mL) in the presence or absence of C-Man-WSPW or WSPW (10 μM). The activation status of NF-κB was examined in the cell lysates by luciferase reporter assay as described in the methods. (C) Cells were pretreated at 37°C for 10 min with or without JNK inhibitor and/or the NF-κB activation inhibitor, and treated for 2 h with LPS (1 μg/mL) in the presence or absence of C-Man-WSPW or WSPW (10 μM), as described above. Then, amount of TNF-α released into the medium was estimated by ELISA for TNF-α.

despite that LPS-induced NFκB signaling was little influenced by C-Man-WSPW or WSPW.

JNK signaling pathway is involved in enhancement of LPS-induced upregulation of TNF-α by C-Man-WSPW

To investigate whether the signaling pathways of JNK and/or NF-κB are involved in the LPS-induced production of TNF-α, levels of TNF-α were examined in cells treated with or without LPS, LPS plus WSPW, and LPS plus C-Man-WSPW in the presence or absence of specific signaling inhibitors. SP600125 (a JNK Inhibitor) and a NF-κB activation inhibitor were used to inhibit the activities of JNK and NF-κB, respectively. In Figure 7A, the cells were pretreated for 10 min with the JNK inhibitor (10 μM), then treated for 15 min with LPS (1 μg/mL) in the presence or absence of C-Man-WSPW or WSPW (10 μM). The activation status of JNK was examined in the cell lysates by immunoblot analysis using the anti-phospho-JNK antibody as described. The results showed that the phosphorylation of JNK was significantly suppressed by the JNK inhibitor in all cases tested. In Figure 7B, after 24 h of transfection with the luciferase vector for NF-κB, the cells were pretreated for 10 min with the NF-κB activation inhibitor (10 μM), then treated for 90 min with LPS (1 μg/mL) in the presence or absence of C-Man-WSPW or WSPW (10 μM). The activation status of NF-κB was examined in the cell lysates by luciferase reporter assay as described. The results showed that the level of transcriptional activity for NF-κB was similarly enhanced by LPS with or without WSPW or C-Man-WSPW, but was suppressed by the inhibitor to approximately 60% of each level of NF-κB activity without the inhibitors. The cells were pretreated for 10 min with or without the JNK inhibitor and/or NF-κB activation inhibitor, and treated for 2 h with LPS, LPS plus WSPW, or LPS plus C-Man-WSPW (Figure 7C). Then, the amount of TNF-α released into the medium was estimated by an ELISA for TNF-α. The results showed that LPS-induced production of TNF-α was suppressed by the inhibitors for JNK and/or NF-κB with or without WSPW or

C-Man-WSPW. Although the suppression was enhanced more with the JNK inhibitor plus NF-κB activation inhibitor, the production of TNF-α was not completely suppressed to the level of the untreated background (see Figure 4A).

Taken together, these results demonstrate that both the JNK and NF-κB pathways are important to the production of TNF-α induced in all cases with LPS, LPS plus WSPW, and LPS plus C-Man-WSPW, suggesting that enhanced JNK signaling in the cells treated with LPS plus C-Man-WSPW may be part of the signaling pathway responsible for upregulating the production of TNF-α.

C-Mannosylated TSR-derived peptides enhance LPS-induced phosphorylation of TAK1 in RAW264.7 cells

In terms of signaling pathways for LPS-induced TNF-α production, we found that the activation of JNK was specifically upregulated by LPS in the presence of C-Man-WSPW. To further investigate how C-Man-WSPW enhances LPS-induced activation of JNK, we focused on the upstream signaling molecules, such as interleukin-1 receptor-associated kinase 1 (IRAK1) and transforming growth factor-β (TGF-β)-activated kinase 1 (TAK1). IRAK1 is one of the upstream serine/threonine kinases activated by LPS through Toll-like receptor 4 (TLR4) (Janssens and Beyaert 2003). The activation of IRAK1 occurs with a concomitant self-phosphorylation of serine or threonine (Kollewe et al. 2004). In Figure 8A, cells were treated with LPS (1 μg/mL) in the presence or absence of C-Man-WSPW or WSPW (10 μM). The activation status of IRAK1 was examined in the cell lysates by immunoblot analysis using the anti-phospho-IRAK1 (Thr-209) antibody, as described. The results showed that the pattern of phosphorylation of IRAK1 did not differ with LPS, LPS plus WSPW, or LPS plus C-Man-WSPW, suggesting that the activation of IRAK1 was little influenced by WSPW or C-Man-WSPW. On the other hand, TAK1, a MAPK kinase kinase (MAPKKK) activated by LPS through TLR4 signaling, is also involved in the LPS-induced signaling pathways to activate NF-κB and MAPKs, such as JNK (Irie et al. 2000; Chen et al. 2002; Silverman et al. 2003) (Figure 8B). The activation of TAK1 occurs with a concomitant autophosphorylation of serine or threonine (Kishimoto et al. 2000; Singhirunnusorn et al. 2005). The activation status of TAK1 was also examined in the cell lysates by immunoblot analysis using the anti-phospho-TAK1 (Thr-187) antibody, as described in Materials and methods. The results showed that the phosphorylation of TAK1 was significantly enhanced by LPS plus C-Man-WSPW, but not by LPS plus WSPW or LPS alone (Figure 8B).

Taken together, C-Man-WSPW or WSPW did not influence the LPS-induced activation of IRAK1, a pivotal LPS-induced kinase located upstream of TAK1, which was consistent with the finding that the quantity of LPS-FITC bound to the cells was not affected by C-Man-WSPW or WSPW. However, C-Man-WSPW specifically upregulated LPS-induced activation of TAK1, a pivotal MAPKKK for LPS-induced activation of MAPK pathways. This was consistent with enhanced LPS-induced activation of MAPKs such as JNK by C-Man-WSPW,

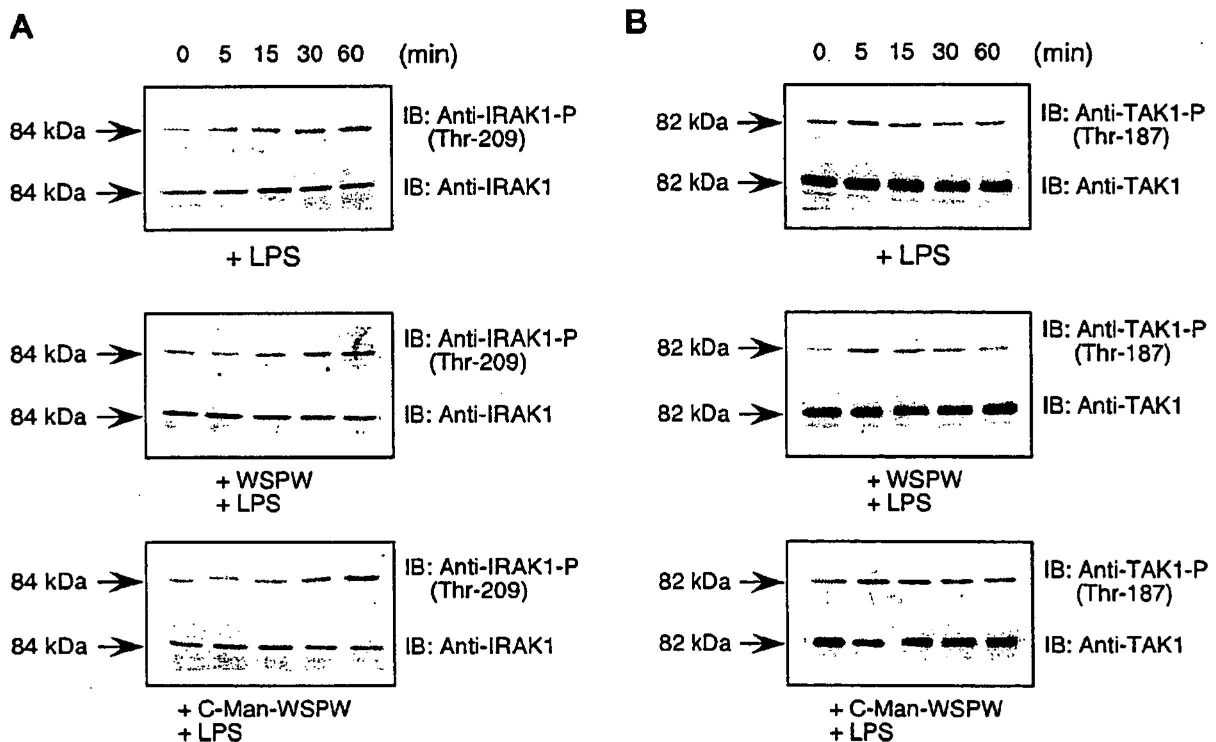

Fig. 8. LPS-induced phosphorylation of TAK1 is highly upregulated in RAW264.7 cells treated with C-Man-WSPW. (A) Cells were treated with LPS, LPS plus WSPW, or LPS plus C-Man-WSPW, as described in Figure 5B. The activation status of IRAK1 was examined in the cell lysates by immunoblot analysis using the antiphospho-IRAK1 (Thr-209) antibody as described in the methods. (B) Cells were treated with LPS, LPS plus WSPW, or LPS plus C-Man-WSPW, as described above. The activation status of TAK1 was also examined in the cell lysates by immunoblot analysis using the antiphospho-TAK1 (Thr-187) antibody, as described in Materials and methods.

resulting in increased production of TNF-α and further LPS-induced cell damage.

Discussion

In the present study, to know how C-mannosylation is involved in the functions of macrophages, we chemically synthesized C-mannosylated peptides such as C-Man-WSPW derived from the TSR motif of human TSP-1, and investigated their effect on LPS-induced signaling in macrophage-like RAW264.7 cells.

LPS, a component of the outer cell membrane of gram-negative bacteria, is a strong stimulator of macrophage/monocytes and shows a clearly inflammatory effect. It is also known that LPS is toxic to several types of cells, such as macrophages and endothelial cells, and that LPS is involved in the pathology of sepsis and septic shock (Akira 2001; Bannerman and Goldblum 2003). In this study, we found that C-mannosylated peptides enhanced LPS-induced damage in RAW264.7 cells, especially in the cases of C-Man-WSP, C-Man-WSPW, and C-Man-WSPWS. However, C-Man-WS, C-Man-W, and mannose had a minor effect compared with other C-mannosylated peptides, and nonglycosylated WSPW also had little effect. These results strongly suggest that C-mannosylated peptides, which contain tripeptides such as WSP, effectively enhance LPS-induced damage, and also that C-mannosylation confers a specific function on WSPW peptides which is commonly seen in the TSR motif of cellular proteins. However, it is not clear yet whether these C-mannosylated peptides can be naturally generated in the cell. In addition, to test functional importance of Pro in C-Man-WSP, we prepared C-Man-WSG and C-Man-WSK, in which Pro was replaced by Gly and Lys, respectively, and examined the effect of the C-mannosylated tripeptides on LPS-induced cell damage. As shown in Figure 2D, both C-Man-WSG and C-Man-WSK moderately enhanced LPS-induced cell damage, suggesting that the effect seems not necessarily dependent on Pro in the specific sequence of WSP.

A variety of C-mannosylated proteins have been identified, and some of these proteins, such as TSP, F-spondin, Interleukin-12, complements, and mucins, mainly function in the extracellular spaces (Furmanek and Hofsteenge 2000). Recently, it has been reported that TSP-1 and 2 are cleaved by ADAMTS1 (*A Disintegrin And Metalloprotease with ThromboSpondin 1*), resulting in the production of functional antiangiogenic peptides (Lee et al. 2006). Furthermore, C-Man-W was detected in human serum or urine from patients of renal diseases (Takahira et al. 2001), suggesting that C-Man-W could be a novel biomarker for renal dysfunction. These results suggest that some of the functional C-mannosylated peptides might be generated in vivo by proteolytic degradation of C-mannosylated proteins. However, it is not clear yet what endogenous C-mannosylated peptides or proteins enhance LPS-induced signaling in macrophages. Thus, further investigation is required to identify such endogenous proteins bearing the C-mannosylated peptides in vivo.

In this study, we showed that the enhanced LPS-induced signaling with C-Man-WSPW was not simply due to the enhanced binding of LPS to cells treated with LPS and C-Man-WSPW, suggesting that the cellular binding of LPS was not directly affected by C-Man-WSPW. On the other hand, we found that the binding of C-Man-WSPWC-biotin to cells was greater than that of biotin or WSPWC-biotin on flow cytometry. In addition, under the conditions with DSP, we found that C-Man-WSPWC-biotin was predominantly bound to some specific proteins of RAW264.7 cells, compared with the lower binding of biotin or WSPWC-biotin. Together, these results strongly suggest that C-Man-WSPW peptides exert an enhancing effect on LPS-induced cytotoxicity by their binding to some specific target proteins in cells, but not by affecting LPS binding to the cells.

The inflammatory effects of LPS are mediated through several secreted factors, such as TNF-α (Guha and Mackman 2001) and nitric oxide (Nathan 1992). We also found that C-Man-WSPW apparently enhanced the production of TNF-α in cells treated with LPS, compared to those treated with LPS plus WSPW or LPS alone. LPS starts the signal transduction by engaging itself with cell surface TLR4 via sequential binding with LPS-binding protein and CD14 (Guha and Mackman 2001). LPS has been shown to initiate multiple intracellular signaling events, such as the activation of NF-κB, and three distinct MAPKs (i.e., ERK1/2, p38-MAPK, and JNK), and these pathways are involved in the LPS-induced upregulation of TNF-α production. In this study, we found that JNK was significantly activated in the cells treated with LPS plus C-Man-WSPW, compared with the cells treated with LPS plus WSPW or LPS alone, although other kinases such as ERK1/2 and p38-MAPK were also slightly upregulated in the activation by C-Man-WSPW plus LPS. On the other hand, the LPS-induced activation of NF-κB was little influenced by C-Man-WSPW or WSPW. Wilson et al. (1999) reported that TSP-1 or TSP-1-derived peptides containing a WSXW motif enhanced CD3-induced activation of MAPK signaling including JNK signaling in T lymphocytes. In addition, Jimenez et al. (2001) reported that TSP-1 inhibits angiogenesis in corneal neovascularization via the activation of JNK signaling. However, it is not clear how the WXXW motif in the TSR is involved in the signaling to JNK.

The proximal signaling molecules involved in the LPS-induced activation of MAPKs have not been fully identified. LPS is thought to activate ERK1/2 through the Ras/Raf-1/MAPKK pathway, p38-MAPK through the activation of MAPK/ERK kinase (MEK)-3, and JNK through the MEKK-1/MEK4 pathway (Swantek et al. 1997; van der Bruggen et al. 1999). In this study, we focused on the upstream signaling of JNK, and examined the effect of C-Man-WSPW on the activation status of some upstream kinases induced by LPS. IRAK1 is one of the upstream kinases associated with the LPS-specific receptor, TLR-4, and is phosphorylated by LPS to be activated (Li et al. 2000). Unexpectedly, LPS-induced phosphorylation of IRAK1 was not influenced by C-Man-WSPW or WSPW, which was consistent with the finding that both C-Man-WSPW and WSPW did not influence the binding of LPS to cells. However, these results suggested that C-Man-WSPW indirectly acts on some intermediate in the LPS-induced signaling pathways to JNK. Next, we examined TAK1, a MAPKKK mainly involved in LPS-induced JNK signaling (Chen et al. 2002; Silverman et al. 2003), and found that LPS-induced phosphorylation of TAK1 was apparently enhanced by C-Man-WSPW but not by WSPW. This suggests that C-Man-WSPW influenced LPS-induced signaling to activate JNK at the level of TAK1 activity, although how the phosphorylation of TAK1 was upregulated by C-Man-WSPW is not known. Although TAK1 is located downstream of the pathway of LPS/TLR4/IRAK1/TNF-α receptor-associated factor (TRAF6) that activates JNK, an alternative pathway for the activation of TAK1 has been identified in cells expressing latent membrane protein 1 (LMP1) derived from Epstein-Barr

virus (Wan et al. 2004). LMP1 does not require myeloid differentiation factor 88 (MYD88), IRAK1, and IRAK4 to engage TRAF6, and selectively utilize TRAF6, TAK1/TAK1-binding protein, and JNK kinases 1 and 2 to activate JNK. Very recently, Uemura et al. (2006) demonstrated that TAK1 is involved in the LMP1 complex and is essential for activation of JNK but not of NF-κB. This suggests that the LPS signaling pathway can be influenced by other modulators, which are not directly involved in the authentic LPS/TLR-4/TRAF6/IRAK/TAK1 pathways, implying that C-mannosylated TSR-derived peptides may act as another modulator of LPS-induced MAPK signaling by mimicking the functions of modulators such as LMP1, although the precise mechanism for the signal transduction by C-Man-WSPW is yet to be clarified.

In macrophages/monocytes, there is a variety of mannose recognizing proteins included in the mannose receptor family (East and Isacke 2002; McGreal et al. 2005). The mannose receptor family is a subgroup of the C-type lectin superfamily and comprises four members; the mannose receptor (MR), the M-type phospholipase A2 receptor, DEC-205 (CD205), and Endo-180. Among the members, MR and Endo-180 have the capacity to bind carbohydrates such as mannose, fucose, *N*-acetylglucosamine, etc., in a Ca^{2+}-dependent manner via their carbohydrate recognition domains. On the other hand, among the C-type lectin receptors, Dectin-1, which recognizes β-linked glucans from fungi, is known to work in collaboration with TLR2 to facilitate inflammatory responses (Brown et al. 2003; Gantner et al. 2003). This also suggests a possible regulation of TLR-related signaling by C-mannosylated peptides via some co-lateral regulatory pathways. Hence, another question raised is whether the C-mannosylated peptides also modulate other receptor signaling pathways in macrophages (i.e., TLR2, TLR6, TLR9, interleukin-1 receptor, and TNF-α receptor), compared with the LPS/TLR4 pathway. Nishikawa et al. (2004) reported that C-Man-W is not recognized in vitro by conventional mannose-binding lectins such as concanavaline A and mannose-binding lectin-C, a lectin abundant in mouse serum. However, it is not clear yet what specific molecule binds to C-Man-W and C-mannosylated peptides. Further investigation to clarify the specific receptors recognizing the motif-containing C-mannosylated peptides is required.

In conclusion, C-mannosylated TSR-derived peptides have an enhancing effect on the cytotoxicity of LPS by increasing the production of TNF-α through enhanced activation of JNK. This study demonstrates a novel regulatory function of the C-mannosylated TSR-derived peptide motif in the innate immunity of macrophages, and also suggests unrevealed functions of C-mannosylated proteins or peptides in macrophages.

Materials and methods

Materials

Antibodies against ERK1/2, phospho-ERK1/2 (Thr-202/Tyr-204), p38-MAPK, phospho-p38MAPK (Thr-180/Tyr-182), JNK, phospho-JNK (Thr-183/Tyr-185), TAK1, phospho-TAK1 (Thr-187), IRAK1, phospho-IRAK1 (Thr-209), and IκBα were purchased from Cell Signaling Technology (Beverly, MA). Peroxidase-conjugated secondary antibodies against IgG of rabbit and mouse were from Dako (Glostrup, Denmark). LPS from *Escherichia coli* O55:B5 and fluorescein isothiocyanate (FITC)-conjugated LPS (LPS-FITC) were obtained from Sigma-Aldrich (St. Louis, MO). Nuclear factor-κB (NF-κB) activation inhibitor [i.e., 6-amino-4-(4-phenoxyphenylethylamino)quinazoline] was from Calbiochem. DSP was from Pierce Biotechnology (Rockland, IL). The other reagents used in the study were all of high grade, from Sigma-Aldrich or Wako pure chemicals (Osaka, Japan).

Chemical synthesis of C-mannosylated peptides and derivatives

C^2-α-D-C-Mannosylpyranosyl-L-tryptophan (C-Man-W) and C-mannosylated TSR-derived peptides (C-Man-WS, C-Man-WSP, C-Man-WSG, C-Man-WSK, C-Man-WSPW, and C-Man-WSPWS) were synthesized essentially, as described previously (Manabe and Ito 1999; Manabe et al. 2003). WSPW peptides, in which the first W has the potential to be C-mannosylated, are derived from TSR2 of human TSP-1, and the first W corresponds to Trp423 in the amino acid sequence (Lawler and Hynes 1986; NCBI Accession number NP_003237) (Figure 1). Silica gel 60N (spherical, neutral, Kanto Chemical Co., Inc, Tokyo) was used for flash column (40–100 μM) and open column (100–200 μM) chromatography. Sephadex™ LH-20 (GE Healthcare Biosciences, Buckinghamshire, UK) was used for size-exclusion chromatography. Silica gel 60 F_{254} (E. Merck, Whitehouse Station, NJ) was used for analytical thin-layer chromatography. MALDI-TOF MS spectra were measured with a Shimadzu AXIMA-CFR using 2,5-dihydrobenzoic acid or α-cyano-4-hydroxycinnamic acid as a matrix. ^{1}H-NMR (nuclear magnetic resonance) spectra were recorded at ambient temperature (23–24°C) in $CDCl_3$, CD_3OD or DMOS using JEOL EX 400 MHz spectrometer. A Waters C18 SepPack Cartridge was used for reverse phase column chromatography (Waters, Milford, MA). Boc amino acids were purchased from Kokusan Laboratory Chemicals, TCI, or NOVA BioChem. The biotin derivative was purchased from Pierce Biotechnology. Other chemicals were purchased from Sigma-Aldrich, TCI, or Kanto chemicals.

Synthesis of C-Man-WSPW Boc-Ser-Pro-Trp-OMe and other peptides were prepared by the conventional solution-phase Boc peptide synthesis protocol. For example, Boc-Pro-Trp-OMe (767 mg, 1.85 mmol) was dissolved in CH_2Cl_2 (20 mL), and 4.0 M HCl (20 mL, dioxane solution) was added. After the mixture had been stirred at room temperature for 2–5 h, the solvent was evaporated. Then, dioxane was added and removed in vacuo several times to remove HCl. The mixture was dissolved in CH_2Cl_2, then diisopropylethylamine (i-Pr_2NEt) (0.48 mL, 2.78 mmol), Boc-Ser-OH (570 mg, 2.78 mmol), and 1-hydroxybenzotriazole (HOBt) (375 mg, 2.78 mmol) were added. Subsequently, water soluble carbodiimide (WSCDI, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide HCl) (530 mg, 2.78 mmol) was added at 0°C. After the mixture had been stirred overnight, the solution was diluted with $CHCl_3$ and an aqueous 10% citric acid solution. The aqueous layer was extracted with $CHCl_3$. The combined layers were washed with brine and dried over Na_2SO_4. After removal of the solvent in vacuo, the residue was purified by silica gel column chromatography ($CHCl_3$:MeOH 9:1–4:1) to give the product.

The Boc group of Boc-Ser-Pro-Trp-OMe (235 mg, 0.468 mmol) was removed, as described above. Then, the residue was dissolved in dimethylformamide (DMF) (1 mL),

and i-Pr_2NEt (0.13 ml, 0.729 mmol), Fmoc-(C-Man)-Trp (229.5 mg, 0.39 mmol), and (7-azabenzotriazole-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate (193 mg, 0.508 mmol) were added at 0°C (Miller et al. 2003). After the mixture had been stirred overnight, the residue was directly purified by size-exclusion chromatography [LH 20 (MeOH as eluent)] and subsequent silica gel column chromatography ($CHCl_3$:MeOH 9:1–4:1) to give Fmoc-(C-Man)-Trp-Ser-Pro-Trp-OMe. MS *m/z* 995 (M^++Na). Fmoc-(C-Man)-Trp-Ser-Pro-Trp-OMe (297 mg 0.201 mmol) was dissolved in MeOH (3 mL) and aqueous 10% NaOH (3 mL) was added. After 1 h, the mixture was purified with a SepPak cartridge (H_2O-H_2O:MeOH 4:1) to give the product. MS *m/z* 759 (M^++Na). (C-Man)-Trp-Ser-Pro and (C-Man)-Trp-Ser were synthesized similarly. (C-Man)-Trp-Ser-Pro; MS *m/z* 596 (M^++Na). (C-Man)-Trp-Ser; MS *m/z* 476 (M^++Na).

C-Man-WSPWC-biotin To a solution of Fmoc-(C-Man)-Trp-Ser-Pro-Trp-Cys(S^tBu)-OMe (55.6 mg, 0.0478 mmol) in MeOH (1 mL), H_2O (0.5 mL), and DMF (0.5 mL), tributylphosphine (PBu_3) (23 mL, 0.0919 mmol) was added and the mixture was stirred at room temperature for 1 h under a N_2 atmosphere. After the solvent was removed in vacuo, the residue was dissolved in DMF (0.5 mL). To the solution, (+)-biotinyl-iodoacetamidyl-3,6-dioxaoctanediamine (27 mg, 0.050 mmol) and i-Pr_2NEt (10 μL, 1.78 mmol) were added and the mixture was stirred overnight. The mixture was purified using LH 20 (MeOH as eluent), then silica gel column chromatography ($CHCl_3$:MeOH 9:1–4:1) to give the product. MS *m/z* 1512. Then Fmoc and methyl ester were removed and purified as described above. MS *m/z* 1276. The purity of C-Man-W containing peptides was determined by 400 MHz ^{1}H-NMR. The purity of other peptides (i.e., non-C-Man-W containing peptides) was determined by both ^{1}H-NMR and HPLC (Itertsil ODS column, water:CH_3CN containing 0.1% TFA). The purity of all peptides was more than 95%.

Cell lines and culture RAW264.7 cells, a clonal line of mouse macrophage-like cells, were obtained from American Type Culture Collection (TIB-71). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM), supplemented with 20% fetal calf serum (FCS) under a humidified atmosphere of 95% air and 5% CO_2 at 37°C.

Cell injury assay The viability of cultured cells was evaluated using MTT, as described (Ihara et al. 2006). The cells (1000–2000) were placed in 100 μL of medium per well in 96-well plates, and cultured overnight. After treatment with LPS for 48 h in the presence or absence of C-Man-W, C-mannosylated peptides, and peptides, 10 μL of 0.5% MTT solution was added, and the cells were incubated at 37°C for 4 h. The reaction was stopped by adding 100 μL of lysis buffer A (10% SDS and 0.1 M HCl), and then cell viability was evaluated by measuring the absorbance at 570 nm using a microplate reader. Dead cells were detected by fluorescent DNA staining using SYTOX Green (Invitrogen) (Cheung et al. 2000). Cultured cells were stained with SYTOX Green (1 μM) for 10 min, fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS; pH 7.2) for 30 min under darkness, and visualized by fluorescence microscopy with excitation at 485 nm and emission at 520 nm using a Carl Zeiss LSM5 microscope (Carl Zeiss, Jena, Germany), and analyzed by PASCAL analytical software.

LPS-FITC binding assay Cells were incubated at 37°C for 20 min in medium containing LPS-FITC (1 μg/mL) in the presence or absence of WSPW or C-Man-WSPW (10 μM). After a wash with PBS, the binding of LPS-FITC to the cells was analyzed using a Cytomics FC500 Flow Cytometry System (Beckman Coulter, Fullerton, CA).

Estimation of C-Man-WSPWC-biotin binding Cells were incubated at 37°C for 20 min in PBS with biotin, WSPWC-biotin, or C-Man-WSPWC-biotin (10 μM). After a wash with PBS, the cells were blocked with 3% bovine serum albumin (BSA) in PBS, incubated with FITC-conjugated Nutr-avidin (Pierce Biotechnology) for 15 min, and washed with PBS containing 1% BSA. After another wash, the binding of biotin conjugates to the cells was analyzed using a Cytomics FC500 Flow Cytometry System. Alternatively, after incubation with the biotin conjugates as described above, the cells were treated for 10 min with or without 1 mM DSP, a membrane permeable cross linker. After quenching with 1 M Tris–HCl (pH7.2), the cells were washed with PBS, and harvested by using a scraper. The cells were lysed in lysis buffer B (20 mM Tris–HCl [pH 7.2], 130 mM NaCl, and 1% NP-40 including protease inhibitors [20 μM APMSF, 50 μM pepstatin, and 50 μM leupeptin]). Protein samples were electrophoresed on 7.5% SDS-polyacrylamide gels under non-reducing conditions and then transferred to a nitrocellulose membrane as described before (Ihara et al. 2005). The membrane was blocked with 5% skim milk in Tris-buffered saline (TBS; 10 mM Tris–HCl [pH 7.5] and 150 mM NaCl) and then incubated at 4°C for 10 min with the peroxidase-conjugated Nutr-avidin (Pierce Biotechnology) in TBS containing 0.1% Tween 20. After a wash with TBS containing 0.1% Tween 20, the blots were developed using an ECL chemiluminescence detection kit (GE Healthcare Biosciences) according to the manufacturer's instructions.

Immunoblot analysis Cultured cells were harvested and lysed in lysis buffer B, as described above. Protein samples were electrophoresed on 7.5–10% SDS-polyacrylamide gels under reducing conditions and then transferred to a nitrocellulose membrane. The membrane was blocked with 5% skim milk in TBS and then incubated at 4°C overnight with the primary antibody in TBS containing 0.1% Tween 20. The blots were coupled with the peroxidase-conjugated secondary antibodies, washed, and then developed using an ECL chemiluminescence detection kit.

JNK activity assay JNK activity was assayed using a JNK assay kit (Cell Signaling Technology) according to the manufacturer's protocol with glutathione S-transferase-c-Jun fusion protein (GST-c-Jun) as a substrate. Phosphorylation of GST-c-Jun was assessed by immunoblot analysis using specific antibody.

TNF-α assay Cells were plated in 96-well plates (10,000 cells/well), and cultured overnight. The medium was renewed. Then, cells were stimulated with LPS (1 μg/mL) in the presence or absence of C-Man-W, C-mannosylated peptides, and peptides (10 μM). After the incubation, the culture medium was collected, and the concentration of TNF-α in the sample was evaluated using a mouse TNF-α ELISA kit according to the manufacturer's instructions (BioSource International, Camarillo, CA). All experiments were done in quadruplicate.

Reverse transcription-polymerase chain reaction (RT-PCR) Total RNA was prepared from cultured cells using standard methods and was reverse transcribed using a One Step RNA PCR Kit (AMV) (TaKaRa Biomedicals, Shiga, Japan) with avian myoblastosis virus-derived reverse transcriptase XL according to the manufacturer's instructions. PCR was run for 19 cycles of 95°C for 0.5 min/65°C for 0.5 min/72°C for 1.5 min. Primer sequences were as follows: For mouse TNF-α (GenBank accession number NM_013693) (Fransen et al. 1985), forward: 5′-TCT CAG CCT CTT CTC ATT CC-3′; reverse: 5′-GTC CCA GCA TCT TGT GTT TC-3′; for mouse β-actin (BC063166), forward: 5′-GAG CTA TGA GCT GCC TGA CG-3′; reverse: 5′-AGC ATT TGC GGT GCA CGA GG-3′.

Luciferase activity assay The PathDetect pNFκB-Luc Cis-Reporter Plasmid, a luciferase reporter construct for the NFκB gene promoter, was obtained from Stratagene (Tokyo, Japan). The vector was introduced into cells by using Lipofectamine2000 (Invitrogen) according to the manufacturer's instructions. After 24 h of transfection, cells were treated with LPS (1 μg/mL) in the presence or absence of WSPW or C-Man-WSPW (10 μM), or left untreated for the periods indicated in the text. Then luciferase activity was assayed with cellular extracts by using a Dual-Luciferase Reporter Assay System (Promega), as described before (Yasuoka et al. 2004).

Statistical analysis Statistical analysis was performed using Student's *t*-test or ANOVA (StatView software). Significance was set at $P < 0.05$.

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Conflict of interest statement

None declared.

Abbreviations

DMEM, Dulbecco's modified Eagle's medium;; DMF, dimethylformamide; DSP, dithiobis[succinimidylpropionate]; ELISA, enzyme-linked immunoabsorbent assay; ERK, extracellular signal-regulated kinase; FCS, fetal calf serum; FITC, fluorescein isothiocyanate; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GST, glutathione S-transferase; IκBα, inhibitor of κBα; i-Pr_2NEt, diisopropylethylamine; IRAK1, interleukin-1 receptor-associated kinase 1; JNK, c-Jun N-terminal kinase; LMP1, latent membrane protein 1; LPS, lipopolysaccharide; MAPK, mitogen activating protein kinase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NF-κB, nuclear factor-κB; PBS, phosphate-buffered saline; RT-PCR, reverse transcription-polymerase chain reaction; TAK1, TGF-β-activated kinase 1; TBS, Tris-buffered saline; TGF-β, transforming growth factor β; TLR, Toll-like receptor; TNF-α, tumor necrosis factor-α; TRAF6, TNF-α receptor-associated factor 6; TSP, thrombospondin; TSR, thrombospondin type 1 repeat.

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「次の百年へ」老化と抗加齢の科学

活性酸素と老化*

近藤宇史†

はじめに

活性酸素とその代謝産物によってもたらされる酸化ストレスは，生体構成成分のタンパク質，脂質や核酸を酸化修飾してそれらの構造と機能を変化させる．これらの酸化修飾物が蓄積すると細胞の機能にも影響を及ぼし，老化が進む．生体はつねに活性酸素に曝露されているほかに，意図的に活性酸素を産生し殺菌や細胞内情報伝達に使用している．一方，体液や各組織細胞はそれぞれ活性酸素を消去する防御機構を有している．活性酸素が過剰に加わったとき，酸化ストレスとして細胞障害が生まれ，そのストレスが長期に亘るときに病的老化のもととなる慢性疾患を進展させる．たとえば，慢性喫煙は肺癌，慢性肺気腫，心血管病変の増悪因子であるが，その機序に酸化ストレスがあると考えられる．肥満，耐糖能異常，高脂血症，高血圧のいずれにも酸化ストレスによる組織障害が存在する．

活性酸素種(reactive oxygen species: ROS)によるタンパク質のチオール基の酸化修飾と，それを還元する因子による酸化還元(レドックス)のバランスは，細胞内情報伝達や遺伝子の発現などの重要な細胞機能を制御している．ROSが細胞障害ばかりでなく，細胞内情報伝達を含めた細胞機能の制御に関連するレドックス制御因子の研究が疾病の予知や予防の面から注目されている．

Key words: Reactive oxygen species, Oxidative stress, Aging, Akt, Redox

*Reactive Oxygen Species and Aging

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1. 活性酸素の生成

生体内で主要なROSは，スーパーオキシドアニオン(O_2^-)，過酸化水素(H_2O_2)，ヒドロキシラジカル(OH)である．ROSの産生は，(1)ミトコンドリア呼吸鎖での電子伝達系，(2)ペルオキソームでの脂肪酸代謝，(3)チロクロームP-450による反応，(4)貪食細胞による“respiratory burst”の際などで行われる．ミトコンドリアでのROS産生は電子伝達系を流れる電子の約0.1-2%程度とされる．ミトコンドリアのMn-スーパーオキシドジスムターゼ(SOD)がH_2O_2に変える．

炎症細胞は，異物貪食をする際にROSのmixtureを放出する．それには，O_2^-，H_2O_2，・OH, hypochlorideが含まれる．そのROSは慢性炎症で病的意義があると考えられる．細胞膜に局在するNADPH (nicotinamide adenine dinucleotide phosphate, reduced) oxidaseは炎症細胞では炎症と関連するほかに，ホルモン，増殖因子やサイトカインなどの受容体を介したシグナルがこの酵素を活性化して微量のROSを産生し，シグナル伝達に関連する．

2. 細胞の抗酸化機構

細胞内小器官は，酸化ストレスに対して独自のアンチオキシダント機能を有している．核ばかりでなく，小胞体も本来のタンパク質の合成と品質管理に対するストレスに独自の反応を示す．ミトコンドリアはアポトーシスを制御するシグナルの担い手であり，高いアンチオキシダントを有してストレスに応答している．たとえばO_2^-を消去するMn-SODが局在している．SODが生成するH_2O_2を消去するグルタチオン過酸化酵素やグルタチオン(GSH)，レドックスに関わるチオレドキシンやグルタレドキシン(GRX)2も存在する．これら小器官のストレス応答の破綻が酸化ストレスによる老化進展をもたらすとするデータも多い．

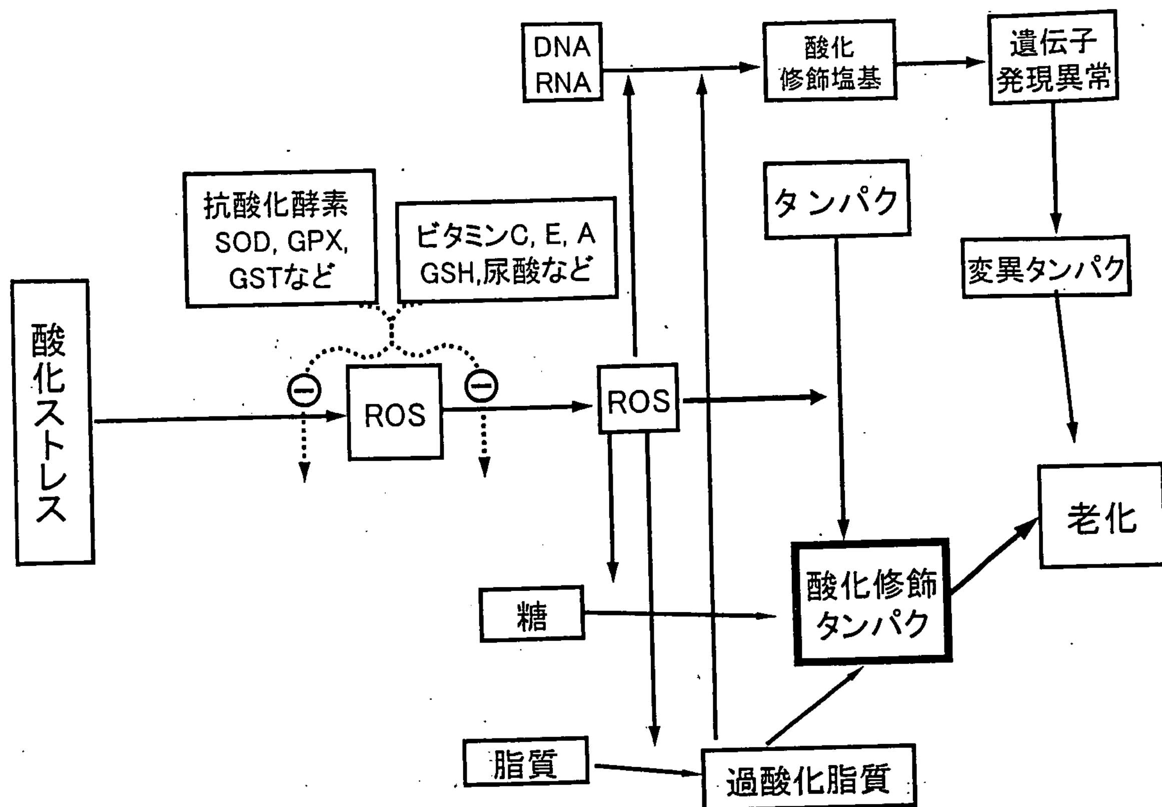

図1　活性酸素種による老化の進展．種々のストレスで細胞内外で生じるROSは，細胞構成成分のタンパク質，脂質，糖質，核酸に酸化修飾を引き起こす．酸化修飾タンパクや遺伝子の異常による変異タンパク質の増加が老化へと結びつく．［文献7より引用，一部改変］

3. 活性酸素の標的因子と測定法

タンパク質，脂質と核酸が酸化修飾されることが，オキシダントが過剰に発生していることの証拠として測定されている．

(1) 脂質過酸化

脂質過酸化物の測定は，脂質過酸化物とタンパク質の付加体に対する特異抗体を作製して免疫学的に測定する方法が開発され，組織細胞や血漿タンパク質での検討が進められている．2-アルケナール，4-ヒドロキシノネナール，4-ヒドロキシ-2-ヘキセナール，イソレブグランジンなどのケトアルデヒドなどの測定方法が確立している．

(2) 核酸の酸化修飾

現在多くの酸化修飾核酸の測定法が開発されている．8-ヒドロキシ-2'-デオキシグアノシン(8-OHdG)などの核酸酸化物を測定する．

(3) タンパクの酸化修飾

カルボニル化タンパク質の免疫学的定量法が一般的に用いられている．カルボニル化タンパクは血漿や組織細胞で多く認められている．

4. 酸化ストレスと細胞の老化

ROSによって修飾されたタンパク質，脂質，核酸が増加して蓄積すると種々の機能障害を生じる(図1)．固体では1日当たりDNAは10^5個の酸化傷害を受けていると言われており，加齢によって核やミトコンドリアでのDNA過酸化物の蓄積が増加する．ROSは直接DNAのstrand breakやcross-linkを形成してmRNA(messenger ribonucleic acid)の転写停止，翻訳のエラーや遺伝子の不安定化を来す．酸化修飾されたタンパク質はクロスリンクしやすいために，プロテアソームでのタンパク分解を受けづらい．酸化LDL(low-density lipoprotein)，過酸化脂質，糖化タンパクや高血圧におけるシアストレスのシグナルも細胞に過剰な酸化ストレスとして働く．脂肪細胞由来のTNF-α(tumor necrosis factor-α)もROS産生に働く．さ

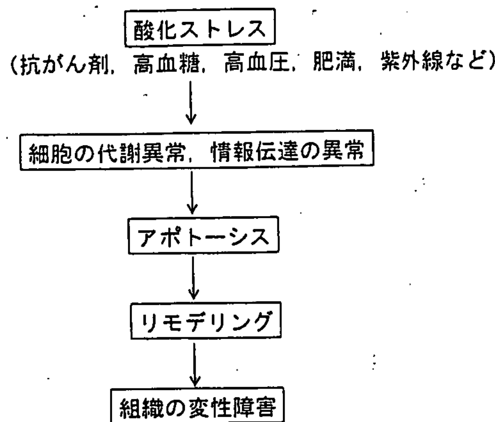

図2 酸化ストレスによる組織細胞のアポトーシスとリモデリング．細胞内外にはアンチオキシダントがあってROSを消去するが，過剰なROSは脂質，タンパク質，DNAを酸化修飾する．その結果心筋のアポトーシスからリモデリングが引き起こされ，組織障害と機能不全の原因となる．高血糖，高血圧，肥満，紫外線，炎症，抗癌剤などが酸化ストレスを生じる．［文献1より引用，一部改変］

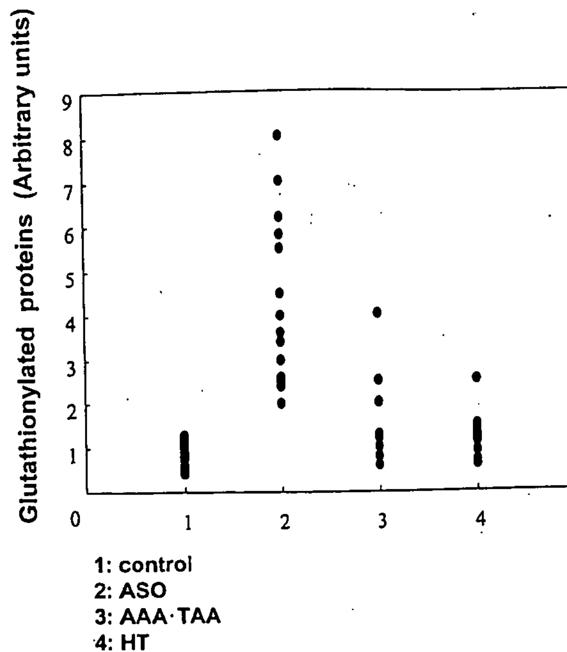

図3 閉塞性動脈硬化症におけるグルタチオン化血清タンパク．患者血清中のレドックス異常で引き起こされるグルタチオン化タンパクを測定．ASO; 閉塞性動脈硬化症，AAA-TAA; 大動脈瘤，HT; 高血圧症．

らに血管内のマクロファージや好中球からのROSも酸化ストレスとなる．動脈硬化病変で酸化修飾タンパクや脂質過酸化物が検出されることから，慢性酸化ストレスが血管の老化を進展させていると考えられる．

心筋障害による心不全，肺気腫による呼吸不全，2型糖尿病などによる組織障害でも，組織細胞に酸化ストレスが負荷されることで細胞のアポトーシスが生じ，組織のリモデリングへ進み，変性と機能不全へと進む(図2)[1]．

呼吸器疾患に関連して，長井らは最近喫煙による酸化ストレスの意味を報告している[2]．すなわち，気管支肺胞洗浄液中の酸化修飾タンパク質を測定してみると，若年者では喫煙の有無による差異は見られなかったが，老年者では慢性喫煙によって非喫煙者とは異なる酸化修飾タンパクの著明な増加を認めた．さらに洗浄液中のグルタチオン(GSH)/酸化型グルタチオン(GSSG)比が低下しており，喫煙によってレドックスにも変化を及ぼすことが示された．

5．レドックス制御と老化

レドックスは細胞機能全般の制御に関連する重要な仕組みである．GSHがROSによってGSSGになるとタンパク質のシステイン残基は，GSSGで修飾され可逆性の -S-S- か -SSG(グルタチオン化)，さらに不可逆性の -SOxH となる．可逆的なシステインの酸化還元状態はその組織細胞や体液でのレドックスバランスを反映することから，特にグルタチオン化されたタンパクの測定は，新しい血管障害危険因子となると考えられる．最近われわれは，血管老化疾患である閉塞性動脈硬化症(ASO)でのグルタチオン化血清タンパクの測定を試みた[3]．ASOでは，グルタチオン化タンパクが上昇しており，他の心血管障害では変化が著明でなかったことから，この測定の意義があると考えられる(図3)．

転写因子による遺伝子発現も，転写因子タンパクのDNA結合を制御するレドックスによって調節されている．情報伝達に働く多くのキナーゼはmRNAの発現，タンパクの発現と，翻訳後の酵素活性の修飾で制御されるが，システインのSH基が酸化型か還元型かによって活性が調節されている．

細胞が消去できなかったROSは直接アポトーシスへと向かうASK-1，JNK，p38MAPKなどのシグナルを活性化する(図4)．一方，ROSはレドックスを働かせる起点となり，細胞内情報伝達の活性化を引き起こすことで抗アポトーシス作用を示す．最近われわれが報告したレドックス調節因子GRXによるシグナル伝達の制御機構の1つを紹介する[4]．ROSを添加した心筋細胞では，アポトーシスを防ぐPI3-kinase/Aktの抗アポトーシスシグナルが働く(図5)．Aktはミトコンドリアにアポトーシスを引き起こすシグナルを伝えるBaxを

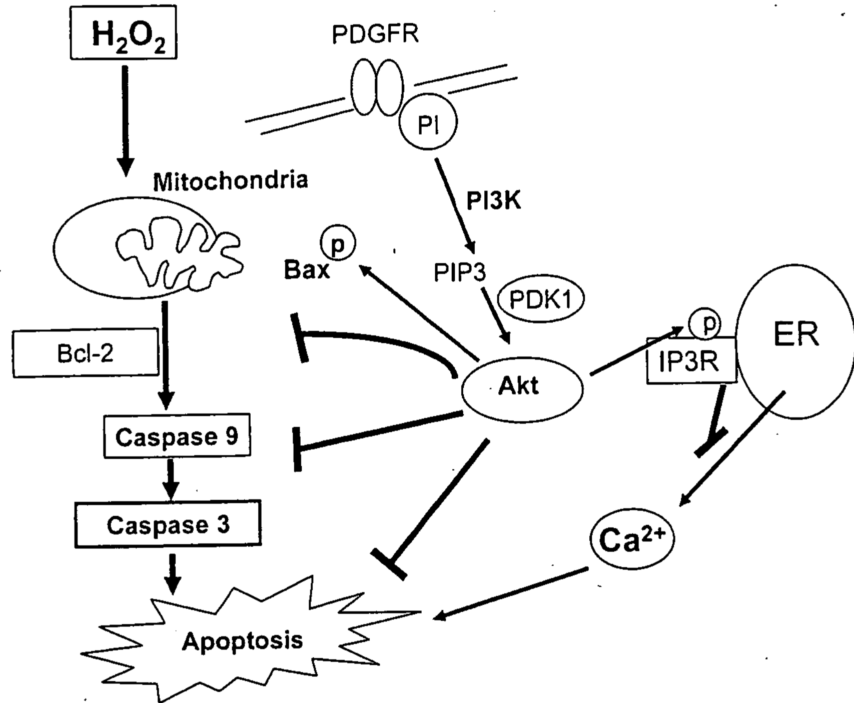

図4 酸化ストレスによるミトコンドリア障害と抗アポトーシスシグナル．増殖因子受容体を介したシグナルはPI3-kinase/Aktの抗アポトーシスシグナルを活性化する．Aktはミトコンドリアからのアポトーシスシグナルを抑制する．また，小胞体(ER)からのアポトーシスに働くカルシウムを，そのポンプであるIP3受容体(IP3R)をリン酸化することで抑制し，抗アポトーシスに働く．

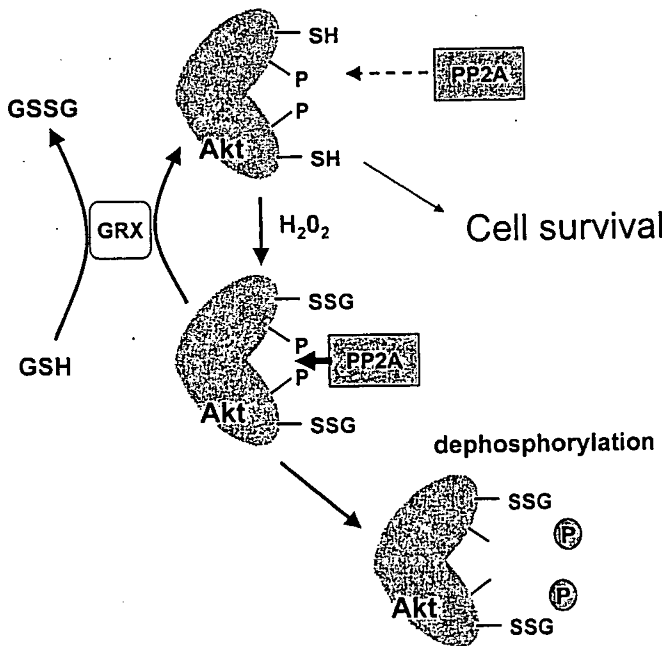

図5 GRXによるレドックス制御．タンパク質のシステイン基(Protein-SH)が，ROSでグルタチオン化(Protein-SSG)されると，GRXはGSHを基質として還元型に戻すように働く．Aktの活性はGSH/GRXシステムで還元型に維持されることで抗アポトーシスシグナルを伝えることが可能となる．

リン酸化することで，ミトコンドリア内への移行を阻害する．さらにAktはミトコンドリアからのアポトーシスシグナル系を抑制して抗アポトーシスに働く．一方，GRXはAktの活性を調節するシステインが還元型であるように働き，その結果リン酸化活性が高く維持される．それは還元型のときにはプロテインフォスファターゼPP2Aが結合しにくいためである．

さらに，性ホルモンによるレドックス制御について女性ホルモン(エストラジオール，E_2)の抗酸化能の検討を行った[5]．E_2は基本的にはE_2受容体(ER)に結合して転写因子として遺伝子発現を調節する．このERにはERαとERβが存在し，性組織細胞ばかりでなく広く組織細胞に発現していることから，E_2の生理的意義は広いことが推測される．ERβを発現している心筋細胞にROSのストレスを与えると，ROSによってアポトーシスシグナルが活性化される．E_2をあらかじめ投与しておいた心筋細胞では，Aktの活性が上昇し，アポトーシスが起こらなくなった．このことは，Aktの活性維持に働くGRXとGSH合成の遺伝子発現がE_2によって誘導された結果である．

インスリンが受容体を介して細胞にシグナルを伝