A Novel Cell-permeable Antioxidant Peptide, SS31, Attenuates Ischemic Brain Injury by Down-regulating CD36*

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Received for publication, October 4, 2006, and in revised form, December 7, 2006 Published, JBC Papers in Press, December 18, 2006 DOI 10.1074/jbc.M609388200

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Oxidative stress is implicated in the pathogenesis of ischemia/reperfusion injury. Recently, we demonstrated that activation of CD36, a class B scavenger receptor, mediates free radical production and tissue injury in cerebral ischemia (1). Oxidized low density lipoproteins (oxLDL) are among the ligands that bind to CD36 and are elevated in acute cerebral infarction. SS31 is a cell-permeable antioxidant peptide that reduces intracellular free radicals and inhibits LDL oxidation/lipid peroxidation (2). The current study was designed to investigate whether treatment with SS31 normalizes ischemia-induced redox changes and attenuates CD36-mediated tissue injury. C57BL/6 mice were subjected to transient middle cerebral artery occlusion (MCAO). Redox status and infarct volume were measured in animals treated with either saline or SS31. Oxidative stress induced by ischemia/reperfusion profoundly depleted glutathione (GSH) concentrations in the ipsilateral cortex and striatum. Treating mice with SS31 immediately after reperfusion significantly attenuated ischemia-induced GSH depletion in the cortex and reduced infarct size. By contrast, the protective effect of SS31 was absent in CD36 knock-out mice, indicating that SS31 is acting through inhibition of CD36. Treating C57BL/6 mice with SS31 reduced CD36 expression in postischemic brain and mouse peritoneal macrophages (MPM). Further in vitro studies revealed that SS31 attenuated oxLDL-induced CD36 expression and foam cell formation in MPM. These in vivo and in vitro studies indicate that the down-regulation of CD36 by novel class antioxidant peptides may be a useful strategy to treat ischemic stroke victims.

Generation of reactive oxygen species (ROS) and depletion of intracellular antioxidants following cerebral ischemia/reperfusion are hallmarks of oxidative stress and lead to tissue injury. Glutathione and ascorbate are major endogenous cerebral antioxidants that serve as biochemical markers of intracellular redox status (3). It is generally accepted that these antioxidants are important for protection of brain during post-ischemic oxidative stress, although dynamic interactions among antioxidants in vivo are not entirely clear. Depletion of intracellular antioxidants has been associated with stroke pathology and depletion of antioxidant status has reduced neuronal damage but has produced inconsistent improvement in stroke outcome (4–8). Ischemic injury also causes a local inflammatory reaction by activated microglia and infiltrated inflammatory cells that further release pro-inflammatory cytokines and ROS within the injured site.

CD36, a glycosylated surface receptor, belongs to a class B scavenger receptor and is localized in lipid rafts of plasma membrane and in mitochondria (9–11). It is expressed on microglia, macrophages, microvascular endothelium, cardiac and skeletal muscle, adipocytes, and platelets and recognizes a variety of ligands including oxidized low density lipoproteins (oxLDL), advanced glycation end products, long chain fatty acids, fibrillar β-amyloid, thrombospondins, and cells undergoing apoptosis (12–14). This multifunctional receptor plays a pivotal role in angiogenesis, inflammation, apoptosis, and lipid metabolism (12, 14). Increased expression of CD36 has been associated with pro-oxidative conditions. Antioxidants, such as α-tocopherol, diminish CD36 expression and reduce the uptake of oxLDL into macrophage (15–17). In addition, a pro-inflammatory response triggered by oxidative stress is abrogated in macrophages from CD36-deficient patients (18), indicating a direct association between manifestations of oxidative stress and CD36 expression.

The mechanism(s) by which post-ischemic inflammation contributes to oxidative stress is not clear. However, a recent study indicates that CD36 contributes to tissue injury in cerebral ischemia (1). In the infarct territory there was increased CD36 expression in microglia/macrophage after ischemia. Compared with the wild-type mice, production of ROS and infarct size was significantly attenuated in mice lacking CD36 (1). These early findings suggest that CD36 may be a target for therapeutic development against cerebral ischemia.

A family of small, cell-permeable antioxidant peptides (SS peptides)3 that readily penetrate the blood brain barrier was

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*This work was supported in part by the Burke Foundation (to S. C.) and National Institutes of Health Grants: NHLBI R01 HL082511 (to S. C.), NIDA P01 DA08924 (to H. H. S.), and NINDS R21 NS048295 (to H. H. S.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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‡§¶To whom correspondence should be addressed: CRF, on behalf of Cornell Research Foundation, Inc. (CRF) for the technology (SS peptides) described in this article. Hazel H. Szeto is the inventor and Sunghee Cho is the co-inventor. CRF, on behalf of Cornell University, has licensed the technology for further research and development to a commercial enterprise in which CRF and Drs. Szeto and Cho have financial interests.
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Described recently (see review in Ref. 19), the SS peptides scavenge ROS and inhibit oxidation of low-density lipoprotein, thereby reducing the generation of oxLDL, ligands for CD36 (2). Unlike other antioxidants, the SS peptides target mitochondria and protect mitochondria against mitochondrial permeability transition, swelling, and cytochrome c release (2) and prevent t-butylhydroperoxide-induced apoptosis (20). These peptide antioxidants are very effective in preventing myocardial stunning and reducing infarct size following cardiac ischemia reperfusion (2, 21–23). To exploit an antioxidant approach by targeting CD36, the present study investigated whether the SS31 antioxidant peptide attenuates cerebral ischemia-induced injury and whether the peptide-induced neuroprotection involves the down-regulation of CD36 expression and function.

**EXPERIMENTAL PROCEDURES**

**Reagents**

SS31 (d-Arg-Dmt-Lys-Phe-NH$_2$; Dmt = 2’,6’-dimethyltyrosine) and SS20 (Phe-d-Arg-Phe-Lys-NH$_2$) were prepared by Dr. Peter W. Schiller (Clinical Research Institute of Montreal, Montreal, Quebec, Canada) using solid phase synthesis as described previously (24). Cu$^{2+}$-oxidized human LDL was purchased from Biomedical Technologies Inc. (Stoughton, MA). Monobasic sodium phosphate was purchased from EM Science (Gibbstown, NJ) and Baker Analyzed HPLC acetonitrile and N,N-dimethylformamide were obtained from Mallinkrodt Baker, Inc. (Phillipsburg, NJ). Unless indicated, all other chemicals were obtained from Sigma.

**Animal Experiments**

All experimental procedures on animals were approved by the Institutional Animal Care and Use Committee of Weill Medical College of Cornell University. C57BL/6 mice were obtained from Charles River Laboratory (Wilmington, MA). A breeding pair of CD36 KO mice was obtained from Dr. Maria Febbraio at Cleveland Clinic Foundation. CD36 KO mice used in the study were backcrossed with C57BL/6 mice six times (98.45% C57BL/6, 1.55% 129/SvJ). The procedures for breeding and genotyping were performed according to a method described elsewhere (25).

**Transient MCAO**

Procedures for MCAO were identical to those previously described (1). Briefly, male mice (22–24 g) were anesthetized with a mixture of isoflurane (1.5–2%), oxygen, and nitrogen. A fiber optic probe was glued to the parietal bone (2-mm posterior and 5-mm lateral to the bregma) and connected to a Laser-Doppler Flowmetry (Periflux System 5010; Perimed, Jarfalla, Sweden) for continuous monitoring of cerebral blood flow (CBF) in the center of the ischemic territory. For MCAO, a 6-0 teflon coated black monofilament surgical suture (Doccol Co, Redlands, CA) was inserted into the exposed external carotid artery, advanced into the internal carotid artery, and wedged into the cerebral arterial circle to obstruct the origin of MCA. The filament was left in place for 30 min and then withdrawn. Only animals that exhibited both a reduction in CBF more than 80% during MCAO and a recovery of CBF >80% by 10 min of reperfusion were included in the study. This procedure leads to reproducible infarcts involving both the cerebral cortex and the striatum (1). Body temperature was kept at 37.0 ± 0.5 °C during both surgical and recovery periods until animals regained consciousness.

**Drug Treatments**

To measure the levels of cysteine, ascorbate and GSH at 6-h post-ischemia, animals were treated intraperitoneally with saline (vehicle), SS31 (2 mg/kg), or SS20 (2 mg/kg) immediately after reperfusion. In initial studies, SS20 was included as a control because it lacks free radical scavenging properties (2). We found a difference in percent GSH change at 6-h post-ischemia between vehicle- and SS31-treated, but not between vehicle- and SS20-treated groups. Therefore, in subsequent studies we utilized only SS31. For measurement of infarct volume, vehicle or SS31 (2 mg/kg or 5 mg/kg of body weight) was administered immediately after reperfusion (0 h), and at 6 h, 24 h, and 48 h post-ischemia. The multiple injection paradigm was used, because there is a biphasic ROS production and sustained CD36 expression in post-ischemic brain (1, 26). The dosage of SS31 used in the present study was based on the dosage previously used in protection against myocardial ischemia (22) and in a model of neurodegeneration (27).

**Measurement of Infarct Volume**

Mice were sacrificed 72 h after ischemia. Brains were removed, frozen, and sectioned (thickness, 30 μm) in a cryostat as previously described (1). Brain sections were collected serially at 600-μm intervals, and stained with Cresyl Violet. Infarct volume was determined using Axiovision (Zeiss, Germany) and the contribution because of swelling was corrected using the method described elsewhere (28).

**Analyses of Cysteine, Ascorbate, and GSH by High Performance Liquid Chromatography (HPLC)**

**Sample Preparation**—Brain tissue from cortex and striatum of both ipsilateral and contralateral side were collected prior to ischemia (sham-operated non-ischemic animals) and 3 h, 6 h, 12 h, and 24 h after 30 min of transient MCAO. Aliquots of right and left cortex and striatum were individually transferred into cryostat vials, snap frozen in liquid nitrogen, and stored at −80 °C until analyses. For determination of selected redox active compounds, each tissue sample was homogenized in 10 volumes of deionized water (w/v) at 4 °C using a Potter-Elvehjem homogenizer fitted with a Teflon pestle attached to a motor. To precipitate proteins, a 100-μl aliquot of each homogenate was added to 400 μl of ice-cold 6.25% (w/v) metaphosphoric acid (MPA), and the sample was incubated for 10 min on ice. After centrifugation at 14,000 rpm for 5 min at 4 °C, aliquots of the 5% MPA supernatant fractions were saved for HPLC determination of redox active analytes by electrochemical detection.

**HPLC Chromatography**—Concentrations of the redox-active ascorbate and the aminothiols, cysteine and GSH, were measured without prior derivatization using a Perkin-Elmer Liquid Chromatograph equipped with an 8-channel coulometric array (CoulArray) detector (ESA, Inc., Chelmsford, MA) (29,
30). The supernatant fractions from the 5% MPA homogenates were injected directly onto a Bio-Sil ODS-5S, 5-μm particle size, 4.0 × 250 mm, C18 column (Bio-Rad) and eluted at ambient temperature with a mobile phase consisting of 50 mM NaH₂PO₄, 0.05 mM 1-octanesulfonic acid, 1% acetonitrile (v/v), and 0.5% N,N-dimethylformamide (v/v) (pH 2.70) at a flow rate of 1 ml/min. PEEK™ (polyetheretherketone) tubing was used and 0.5% injection valve with a 5-cell, respectively, from any particulate matter. A Rheodyne TRI reagent (MRC, Cincinnati, OH). 1 ng (brain tissue) or 200 ng (MPM) total RNA was reverse-transcribed using oligo(dT) primers and the SuperScript First-Strand Synthesis System (Invitrogen), according to the manufacturer’s protocol. PCR primers and probes specific for CD36 and β-actin were obtained as TaqMan predeveloped assay reagents for gene expression (Applied Biosystems, Foster City, CA). β-Actin was used as an internal control for normalization of samples. The PCR reaction was performed using TaqMan Universal PCR Master mix, No AmpErase UNG, and Applied Biosystems 7500 Fast Real-Time PCR system (Applied Biosystems) according to instructions provided by the manufacturers. Reactions were performed in a total volume of 20 μl and were incubated at 95 °C for 10 min followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C. Reactions without cDNA were included as controls. The results were analyzed by 7500 Fast Real-Time PCR Systems software.

**Western Blot Analysis of CD36 in MPM**

Thioglycolate-elicited MPM were harvested and plated in a 6-well culture dish (5 million cells/well) and incubated for overnight with macrophage serum-free media according to a published procedure (31). The cells were then treated with 25 μg/ml oxLDL with or without SS31 (10 nM) for 48 h. MPM were homogenized in three volumes of radioimmune precipitation assay buffer (50 mM Tris-Cl, pH 8.0, 150 mM NaCl, 0.01 μM NaF, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM EDTA, 1% Nonidet P-40) with freshly added protease inhibitor. The homogenate was centrifuged at 10,000 rpm for 5 min at 4 °C, and the protein concentration was determined (Bio-Rad). CD36 protein expression was determined according to the method described previously (1). Proteins (2 μg) were loaded on a gel, electrophoresed, and transferred to polyvinylidene difluoride filters using an electroblotting apparatus. Filters were treated for 1 h in TBS (pH 7.2) containing 0.1% Tween-20 and 5% dry milk, and then incubated with CD36 polyclonal antibodies (1:1000; AF2519; R&D Systems, Minneapolis, MN) followed by mouse IgA-HRP (Sigma). For normalization, anti-β-actin antibody (1:1000, SC1615, Santa Cruz Biotechnology) was used. Filters were washed, and the bands were visualized using a chemiluminescence detection system (Pierce).

**In Vitro Foam Cell Assay**

Thioglycolate-elicited MPM were cultured in 8-well chamber slides. After an overnight incubation in macrophage serum-free media, cultures were treated with 25 μg/ml oxLDL with or without SS31 (10 nM) for 48 h. Cultures were washed with PBS, fixed in methanol for 5 min, stained with oil red-O for 4 h in a moisturized chamber, washed three times with PBS, and covered slipped. The experiments were repeated four times. To quantify the foam cell formation, four areas (625 μm² each) were blindly selected from each culture. Areas that fell within threshold intensity by oil red-O staining were determined using Adobe Photoshop. Values were expressed as total pixels ± S.D.

**RESULTS**

Ischemia Reduces Ascorbate and GSH Levels—Cysteine, ascorbate, and GSH levels were determined in the post-ischemic cortex and striatum. Whereas cysteine levels were gradually decreased in the non-ischemic hemispheres after ischemia, cysteine levels in the ischemic side were significantly higher than those in the contralateral side by 6 h after ischemia (Fig. 1, A and B). By contrast, the levels of ascorbate and GSH, the major water-soluble intracellular antioxidants in brain, were progressively decreased in the ipsilateral side within a few hours of reperfusion (Fig. 1, C–F). Antioxidant depletion was significant in both cortex and striatum by 6 h of reperfusion. The depletion was greater at 24 h, at a time when the infarct is histologically visible. These data suggest that biochemical changes toward pro-oxidation status precede histological changes.
Treatment with SS31, but Not SS20, Attenuates GSH Depletion in Post-ischemic Cortex—To test the efficacy of SS31 on redox status, cysteine, ascorbate, and GSH levels were determined 6 h after ischemia in mice that were treated intraperitoneally (intraperitoneal) with saline (vehicle), SS31 (2 mg/kg), or SS20 (2 mg/kg) upon reperfusion (Table 1). The values were expressed as percent increase (cysteine) and percent depletion (ascorbate and GSH) compared with the contralateral values. No significant percent difference was observed in cysteine and ascorbate among vehicle-, SS31-, and SS20-treated groups (data not shown). By contrast, ischemia-induced percent GSH depletion in the ipsilateral cortex was significantly attenuated in SS31-treated animals compared with that of the vehicle-treated group (Fig. 2). The degree of ipsilateral GSH depletion in SS20-treated mice was not significantly different from that of vehicle-treated mice (Fig. 2). The data suggest that SS31 assists in maintaining antioxidant status and protects against ischemia-induced depletion of GSH in the cortex.

Treatment with SS31 Peptide Reduces Infarct Size—We next addressed whether the decrease in GSH depletion by SS31 is associated with neuroprotection. Mice were subjected to 30 min of MCAO and then treated with vehicle or SS31 (2 and 5 mg/kg, intraperitoneal) upon reperfusion, and at 6, 24, and 48 h after MCAO. Infarct volumes were determined 72 h after ischemia. Low and high dose treatment with SS31 resulted in respective 32 and 36% reduction in infarct volume (p < 0.05, Fig. 3). The degrees of cerebral blood flow (CBF) reduction during MCAO among three groups were similar (vehicle, 85.9 ± 1.1%; SS31 (2 mg), 87.1 ± 1.6%; SS31 (5 mg), 85.3 ± 1.3%). In addition, CBF reperfusion at 10 min was not different among groups (vehicle, 103.1 ± 6.2%, SS31 (2 mg), 109.2 ± 6.2%, SS31 (5 mg), 109.2 ± 5.3%), excluding the possibility that SS31 might alter CBF during the early reperfusion period.

SS31 Does Not Exert Neuroprotective Effects in CD36 KO Mice—CD36 has been shown to mediate ischemia-induced tissue injury (1). To investigate whether SS31 exerts its anti-oxidative property by inhibiting CD36 pathways, GSH levels were measured 6 h after ischemia in CD36 KO mice treated with either vehicle or SS31 (2 mg/kg, intraperitoneal) upon reperfusion. Unlike C57BL/6 mice shown in Fig. 2, SS31 had no effect on ischemia-induced GSH depletion in CD36 KO mice (Fig. 4A). Similarly, SS31 (2 mg/kg) treatment did not attenuate infarct volume in CD36 KO mice (Fig. 4B). Lack of efficacy of SS31 in CD36 KO mice suggests that SS31-induced neuroprotection observed in C57BL/6 mice may be mediated via inhibition of CD36-mediated pathways.

Treatment with SS31 in Mice Attenuates Ischemia-induced CD36 Expression—To investigate the effect of SS31 on CD36 expression in vivo, we determined CD36 gene expression in the SS31-treated ischemic brain. Compared with the contralateral side, CD36 gene expression was significantly increased in the ipsilateral side 24 h after ischemia (Fig. 5A). SS31 treatment resulted in a small but significant reduction in CD36 mRNA levels (Fig. 5A). However, we were unable to detect a difference in CD36 protein expression between vehicle and SS31-treated groups (data not shown). This may be because of the fact that CD36 is expressed mainly in microglia and infiltrating macro-

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**TABLE 1**

<table>
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<th>Treatment</th>
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<th>Ascorbate</th>
<th>GSH</th>
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<tr>
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<td>Ipsilateral</td>
<td></td>
</tr>
<tr>
<td>Cortex</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
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<td>5.83 ± 3.24</td>
<td>44.49 ± 4.20</td>
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<td>41.48 ± 0.91</td>
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</tr>
<tr>
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<td></td>
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</tr>
<tr>
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<tr>
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<td>5.83 ± 2.21</td>
<td>33.39 ± 1.94</td>
</tr>
</tbody>
</table>

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a n = 4–6 animals per group.

b p < 0.05 versus corresponding contralateral side. Student’s t test.
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**FIGURE 2.** Effect of SS peptides on ischemia-induced GSH changes. C57BL/6 mice were subjected to 30 min of MCAO and treated with saline (Veh), SS31 (2 mg/kg body weight) or SS20 (2 mg/kg body weight) peptide immediately after reperfusion. Mice were sacrificed at 6 h post-ischemia. Values are expressed as GSH percent depletion in ipsilateral compared with contralateral side. Error bars indicate S.D. (n = 4–6 animals per group). Note that a difference was observed in percent GSH depletion only in SS31-treated cortex. *, p < 0.05 versus vehicle treated group (Veh), one-way ANOVA with post-hoc Newman-Kuels test. Contral, contralateral side; Ipsil, ipsilateral side.

**FIGURE 3.** Effect of SS31 peptide on ischemia-induced infarct volume in C57BL/6 mice. Mice were subjected to 30 min of MCAO and treated with saline (Veh), 2 mg/kg body weight SS31 (SS31(2)) or 5 mg/kg body weight SS31 (SS31(5)) immediately after reperfusion, 6, 24, and 48 h. Infarct volumes were estimated at 72-h post-ischemia from 12 serial sections (600-μm apart) per animal. Error bars indicate S.D. (n = 11–13 per group), *, p < 0.05 from vehicle-treated group (Veh), one-way ANOVA with post-hoc Newman-Kuels test.

**FIGURE 4.** No protective effect of SS31 on GSH level and infarct size in CD36 KO mice. CD36 KO mice were subjected to 30 min MCAO. A, mice were treated with either saline (Veh) or SS31 (2 mg/kg body weight) immediately after reperfusion, and GSH measurement was performed at 6-h post-ischemia. Values for GSH measurement are expressed as percent GSH depletion in the ipsilateral side compared with contralateral side. Error bars indicate S.D. (n = 4 per group). B, mice were treated with either vehicle or SS31 (2 mg/kg body weight) immediately after reperfusion, 6, 24, and 48 h. Infarct volumes were measured at 72-h post-ischemia. Error bars indicate S.D. (n = 7 per group). No difference was observed between vehicle- and SS31-treated groups.

**FIGURE 5.** Effect of SS31 on ischemia-induced CD36 expression. C57BL/6 mice were subjected to 30 min MCAO and treated with saline (Veh) or SS31 (5 mg/kg body weight) immediately after reperfusion and again 6 h after ischemia. A, for CD36 gene expression, total RNA was prepared from both hemispheres 24 h after ischemia, and CD36 mRNA level was determined. Error bars indicate S.D. (n = 7). *, p < 0.05 versus contralateral side; #, p < 0.05 versus ipsilateral vehicle-treated group, one-way ANOVA with post-hoc Newman-Kuels test. B, correlation analysis of CD36 protein levels and infarct size. CD36 protein levels from SS31-treated MPM were expressed as arbitrary units. SS31 (5 mg/kg) was given at 0, 6, 24, and 48 h, and MPM were harvested 72 h after ischemia. Infarct volume was determined at 72 h after ischemia. CD36 protein levels were normalized against β-actin levels. Note that CD36 protein level is positively correlated with infarct size (r = 0.6390, p = 0.0055).

phases (1), and Western blot analyses may be inadequate to detect SS31-induced reduction of CD36 protein expression that occurs in small percentage of microglia/macrophages in the brain. To confirm SS31 effect on CD36 protein expression, we assessed the relationship between the neuroprotective effect of SS31 (shown in Fig. 3) and CD36 protein expression in macrophages. Correlation analysis within SS31-treated mice revealed that CD36 protein levels in MPM harvested 3 days after ischemia were significantly correlated with infarct size (r = 0.65, p = 0.0055, Fig. 5B), suggesting a functional relationship between neuroprotection and reduced CD36 protein levels by SS31 administration.

**SS31 Attenuates oxLDL-induced CD36 Expression and Function in MPM**—To examine the effect of SS31 on oxidatively stressed inflammatory cells, oxLDL-induced CD36 expression and functions were determined in MPM in the presence and absence of SS31. Compared with control, MPM treated with 25 μg/ml oxLDL for 6, 24, and 48 h increased CD36 mRNA levels by 17.1, 29.4, and 69.0%, respectively. Co-treatment with SS31 in both high (1 μM) and low (10 nM) doses for 48 h significantly attenuated oxLDL-induced CD36 gene expression (Fig. 6A). In
addition, oxLDL-induced CD36 protein expression was also attenuated by SS31 treatment (Fig. 6B). In order to determine whether the reduced CD36 expression by SS31 resulted in less lipid uptake into MPM, in vitro foam cell formation was analyzed. Compared with controls, treating MPM with 25 μg/ml oxLDL for 48 h resulted in increased foam cell formation, which was significantly attenuated by SS31 co-treatment (Fig. 7). Taken together, the data indicate that SS31 attenuates oxLDL-induced CD36 expression and function in MPM.

**DISCUSSION**

Oxidative stress has been implicated in ischemia-reperfusion injury. The success of antioxidant strategies depends largely upon timely administration and the rate of penetration of the antioxidant into the site of tissue injury. The present study demonstrates the efficacy of a cell-permeable antioxidant peptide, SS31, on mitigating cerebral ischemia-reperfusion injury when administered during reperfusion after transient cerebral ischemia. Furthermore, our results suggest that the neuroprotective action of SS31 is mediated through inhibition of CD36 pathways.

Disruption of the balance between pro- and anti-oxidative status occurs during ischemia-reperfusion. In this study, the imbalance of redox status triggered by ischemia-reperfusion stress was investigated by determining the levels of GSH, ascorbate, and cysteine. GSH and ascorbate levels were progressively decreased in the ipsilateral hemisphere with more pronounced reduction in GSH level (Fig. 1, C–F). Resistance to ascorbate depletion may be associated with de novo hepatic synthesis of ascorbate in mice coupled with high expression of the sodium-dependent vitamin C transporter (SVCT) 2 in neurons and choroid plexus in brain (32). Because humans do not synthesize ascorbic acid, GSH depletion in human brain would have a more pronounced impact on balancing redox homeostasis during ischemic injury. Profound depletion of both antioxidants observed in mice at 24-h post-ischemia may reflect cell death at this time. These observations suggest that GSH and ascorbate...
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are early biochemical markers that reflect changes in redox status.

In the present study, SS31 significantly attenuated ischemia-induced GSH depletion and assisted to maintain redox balance in the postischemic cortex, but not in the striatum (Fig. 2) and significantly reduced infarct size (Fig. 3). Since the striatum represents the core of the infarct, administration of antioxidants during reperfusion may be less effective in rescuing the necrotic core. The attenuation of ischemia-induced GSH depletion in the cortex is consistent with the ability of SS31 to scavenge ROS (35). Previous studies have shown that SS31 selectively targets the inner mitochondrial membrane and dose-dependently inhibits mitochondrial permeability transition induced by Ca^{2+} and inorganic phosphate (2). SS31 and related analogs can scavenge H_2O_2, hydroxyl radical and peroxynitrite in vitro (2, 19). Tyrosine can scavenge oxyradicals forming relatively unreactive tyrosyl radicals, which can be followed by radical-radical coupling to give dityrosine, or react with superoxide to form tyrosine hydroperoxide (33). The substitution of phenylalanine for dimethyltyrosine eliminates all scavenging ability in SS20 (2, 19). Unlike SS31, administration of SS20 upon reperfusion did not produce a significant effect on maintaining cortical GSH levels (Fig. 2).

Focal ischemia is associated with a local inflammatory reaction that contributes to tissue damage. In particular, microglia/macrophages become activated and release proinflammatory mediators and ROS. Our previous investigations demonstrated that CD36 expression is increased in microglia/macrophages in the post-ischemic brain and that CD36-mediated ROS production is involved in ischemia-induced brain injury (1). The study also showed that CD36 KO mice were protected against cerebral ischemia by exhibiting significantly reduced infarct volume (1). Because cerebral ischemia-reperfusion produces several CD36 ligands, including long chain fatty acids, Aβ, oxidized LDL, and thrombospondin-1 (34–38), increased receptor-ligand interaction would occur during ischemia-reperfusion. Interestingly, SS31 did not further attenuate ischemia-induced GSH depletion in cortex as well as infarct volume in CD36 KO mice (Fig. 4). Therefore, the lack of efficacy of SS31 on CD36 KO mice in the present study strongly suggests that the neuroprotective action of SS31 in C57BL/6 mice occurs via inhibition of CD36 pathways.

Unlike the previous study that shows a significant protection in CD36 KO mice (1), only a modest reduction in infarct volume in vehicle-treated CD36 KO mice was observed. In the previous study infarct volume was compared between CD36 KO and corresponding wild type mice. Both lines were generated by backcrossing 6 times to C57BL/6 and have identical genetic background (98.45% C57BL/6, 1.55% 129/Sv). By contrast, the efficacy of SS31 was tested in C57BL/6 (100%) mice. In addition, interexperimental comparison from Figs. 3 and 4 and increasing ischemic duration to 30 min in current study may all contribute to exhibit only a moderate reduction in infarct volume in the vehicle-treated CD36 KO mice (Fig. 4) compared with vehicle-treated C57BL/6 mice (Fig. 3).

Although the present study does not reveal the precise mechanism, it provides several possible explanations as to how SS31 attenuates CD36-mediated injury in cerebral ischemia. We previously showed that CD36 expression is up-regulated in the postischemic brain (1). Several studies demonstrate that oxidative stress increases CD36 expression and oxLDL uptake and that antioxidants can reduce its expression and function (15–17, 36). In the current study, CD36 gene expression was significantly lower in SS31-treated ischemic brain 24 h after MCAO, providing evidence that SS31 works, at least in part, by inhibition of CD36 expression. However, we were unable to demonstrate a reduction in CD36 protein in the SS31-treated ischemic brain. Because CD36 protein expression increases primarily in microglia/macrophages in post-ischemic brain (1), it is likely that the sensitivity of Western blot may not be sufficient to detect a difference in protein levels from a subset of cells found in infarct territory. When we determined CD36 protein levels in MPM from SS31-treated ischemic mice, infarct size was positively correlated with CD36 protein levels in MPM (Fig. 5), supporting the view that SS31 attenuates CD36 protein expression. The proposal that SS31 reduces CD36 expression is further supported by an in vitro study showing that SS31 can reduce oxLDL-induced CD36 expression and function in MPM (Figs. 6 and 7).

SS31 treatment may also alter ligand levels, ligand-receptor interaction, and/or downstream signaling pathways during ischemia-reperfusion. In addition to reducing CD36 expression, SS31 can reduce ligand levels by inhibiting LDL peroxidation, as demonstrated in a previous study (2). The receptor/ligand interaction markedly induces further CD36 gene and protein expression (39) in a peroxisome proliferator-activated receptor-γ (PPAR-γ)-dependent manner (40–42) and CD36 gene induction results in a positive forward loop that can further promote oxLDL uptake. If SS31 acts at either the ligand level resulting in reduced oxLDL generation, or the receptor level by reducing CD36 expression, the feed forward loop would be interrupted, thereby reducing CD36 activity. Our results showing no protective effect of SS31 in mice lacking CD36 receptor are consistent with the view of the disruption of the feed forward loop in these mice.

Alternatively, SS31 may act on a downstream event in the CD36 signaling pathway. The CD36-initiated signaling cascade mediates ROS production in macrophages/monocytes in response to oxidative stress (1, 43, 44). The cellular sources of ROS are likely extracellular via activation of NADPH oxidase or from the mitochondrial electron transport chain. Although SS31 can scavenge extracellular ROS, its ability to scavenge mitochondrial ROS would be much more efficient simply because of the fact that SS31 concentrates within the inner mitochondrial membrane (2). CD36 is present in purified mitochondria from skeletal muscle and its expression is regulated by skeletal muscle activity (10, 45). CD36 is also known as fatty acid translocase (FAT) and is involved in uptake and oxidation of long chain fatty acids. It has been shown that free fatty acids (FFA) have adverse effects by increasing ROS production in postischemic heart (46). In cerebrospinal fluid of stroke patients, FFA concentrations are elevated and the elevation is associated with a worse outcome in stroke patients (37, 47). The localization of mitochondrial CD36 in brain cells or whether its expression in mitochondria is regulated by ischemia-reperfusion or oxidative stress is not known. A possible role for SS31 on
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mitochondrial CD36 coupled with FFA uptake and the redox regulation through ROS remains to be investigated.

The presence of mitochondrial CD36, coupled with the ability to translocate FFA into the matrix for fatty acid oxidation, may suggest another possible role of CD36 in ischemia. AMP kinase (AMPK) is considered an energy sensor and becomes activated when the AMP to ATP ratio increases (e.g. ischemia). Therefore, the report that inhibition of ischemia-induced AMPK activation provided neuroprotection in stroke (48) seems paradoxical, provided AMPK activation is to increase ATP production to compensate for ATP loss. Recent studies, however, suggest that prolonged AMPK activation increases ATP usage in the brain. A novel cell-permeable antioxidant peptide, SS31, is effective in attenuating oxidative stress and ischemia-reperfusion cardiac myocytes (49). Interestingly, hypoxia induces translocation of CD36 from the intracellular pool to the plasma membrane for increased uptake of FFA into cells and decreases fatty acid oxidation (50). Further studies on the effects of SS31 on alteration of FFA uptake and fatty acid oxidation would be needed.

In summary, the present study demonstrates that ischemiareperfusion induces a significant shift toward pro-oxidative status in the brain. A novel cell-permeable antioxidant peptide, SS31, is effective in attenuating oxidative stress and ischemia-induced injury. The neuroprotective effect of SS31 antioxidant peptide occurs at the level of CD36 by inhibiting its expression and function, primarily through infiltrating inflammatory cells in the injury site. The present study suggests that targeting CD36 by this novel class of antioxidants can be used as a potential therapeutic strategy to reduce acute stroke-induced injury.

REFERENCES

SS31-induced Neuroprotection via CD36 Down-regulation

A Novel Cell-permeable Antioxidant Peptide, SS31, Attenuates Ischemic Brain Injury by Down-regulating CD36
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doi: 10.1074/jbc.M609388200 originally published online December 18, 2006

Access the most updated version of this article at doi: 10.1074/jbc.M609388200

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