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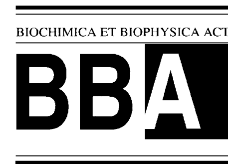

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Review

S100B-stimulated NO production by BV-2 microglia is independent of RAGE transducing activity but dependent on RAGE extracellular domain

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Abstract

The Ca^{2+}-modulated protein, S100B, is expressed in high abundance in and released by astrocytes. At the low levels normally found in the brain, extracellular S100B acts as a trophic factor, protecting neurons against oxidative stress and stimulating neurite outgrowth through its binding to the receptor for advanced glycation end products (RAGE). However, upon accumulation in the brain extracellular space, S100B might be detrimental to neurons. At relatively high concentrations, S100B stimulates NO release by microglia in the presence of lipid A or interferon-γ (IFN-γ). We analyzed further the S100B–microglia interaction to elucidate the molecular mechanism by which the protein brings about this effect. We found that S100B increased NO release by BV-2 microglia by stimulating reactive oxygen species (ROS) production and activating the stress-activated kinases, p38 and JNK. However, S100B stimulated NO production to the same extent in microglia overexpressing a transduction-incompetent mutant of RAGE and in microglia overexpressing full-length RAGE, with a significantly smaller effect in mock-transfected microglia. This suggests that the RAGE transducing activity has little or no role in S100B-stimulated NO production by microglia, whereas RAGE extracellular domain is important, probably serving to concentrate S100B on the BV-2 cell surface. On the other hand, S100B stimulated NF-κB transcriptional activity in BV-2 microglia in a manner that was strictly dependent on RAGE transducing activity, pointing to additional, RAGE-mediated effects of the protein on microglia that remain to be investigated.
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Keywords: S100B; Microglia; Nitric oxide; RAGE

1. Introduction

Besides regulating several intracellular activities by interacting with a relatively large number of target proteins, the Ca^{2+}-modulated protein of the EF-hand type, S100B, also accomplishes extracellular functions [1,2]. S100B is expressed in high abundance in the nervous system where it is localized to astrocytes and several neuronal populations [1,2]. Astrocytes release the protein by an unknown mechanism [3], and S100B release can be regulated by a number of factors [4–6]. At the low levels normally found in the brain extracellular space, S100B acts as a neurotrophic factor protecting neurons against noxious stimuli and stimulating neurite outgrowth [7–15]. These effects depend in part on S100B interaction with the receptor for advanced glycation end products (RAGE) [16]. RAGE is a multiligand receptor belonging to the immunoglobulin superfamily, which has been shown to transduce inflammatory stimuli and effects of neurotrophic and neurotoxic factors, to have a role in tumor growth [17,18], and, as shown recently, to accelerate myogenesis [19]. RAGE engagement has been shown to activate signaling pathways such as Ras-MEK-ERK1/2, p38 MAPK, JNK, Rac/Cdc42 and PI-3K [19–23]. Characteristically, RAGE is expressed in several cell types during development, is repressed or down-regulated at completion of development and is re-expressed in adulthood in certain pathological conditions, including neurodegeneration, inflammation and cancer [17,18]. RAGE engagement in neurons and microglia has been shown to result in nuclear translocation and activation of the transcription factor, NF-κB [16,20,24]. Also, binding of S100B to RAGE in neurons

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has been shown to result in the up-regulation of the anti-apoptotic factor, Bcl-2, in a Ras-MEK-ERK1/2-NF-κB-dependent manner [16], which strongly supports the notion that S100B might protect neurons against noxious stimuli and promote neuronal repair during the very early phase following brain insults and might contribute to neuronal differentiation during development.

However, a deleterious effect of S100B has been proposed to occur during late phases following brain insults, in Down's syndrome (the S100B gene is located in chromosome 21q22.3 [25]) and Alzheimer's disease (for review see Refs. [1,2,26,27]). Acute and persistent activation of RAGE in neurons by high doses of S100B causes neuronal death by apoptosis, as a result of increased production of reactive oxygen species (ROS) [16]. Accumulation of S100B in the brain extracellular space might be a consequence of increased S100B release, decreased clearance or leakage from damaged astrocytes and/or of changes in the composition of the extracellular matrix with, possibly, enhanced adhesion of the protein to extracellular matrix components. However, RAGE might not be the sole S100B receptor [28,29], and high levels of extracellular S100B might be neurotoxic by altering the cytosolic Ca^{2+} concentration in neurons [30] and stimulating nitric oxide (NO) production by astrocytes [31] and microglia, the brain resident macrophages [32,33]. Interestingly, S100B is unable to stimulate NO production by microglia in the absence of co-factors such as lipid A or interferon-γ (IFN-γ) [32,33], which suggests that S100B acts by synergizing with these factors.

We have previously reported that in the presence of IFN-γ, S100B up-regulates iNOS expression and stimulates NO production by the BV-2 microglial cell line in a dose-dependent manner [33]. Here we show that the effect of S100B on NO production by BV-2 microglia is mediated by ROS production and p38 mitogen-activated protein kinase (MAPK) and c-Jun NH_2 terminal protein kinase (JNK) activation. Furthermore, in order to understand the role RAGE in these S100B-mediated effects, we generated BV-2 clones stably overexpressing either full-length RAGE (BV-2/RAGE microglia) or a RAGE mutant lacking the cytoplasmic and transducing domain (BV-2/RAGEΔcyto microglia), while mock-transfected BV-2 cells (BV-2/mock microglia) were used as a control. We show that S100B-mediated NO production by BV-2 microglia is independent of RAGE signaling, although RAGE extracellular domain has an important role.

2. Materials and methods

2.1. Protein purification

Recombinant S100B was expressed and purified as reported [8,34]. S100B was passed through END-X B15 Endotoxin Affinity Resin column (Associated of Cape Cod) to remove contaminating bacterial endotoxin.

2.2. Cell line

The BV-2 microglial cell line was obtained and characterized as described [33,35,36]. Cells were cultivated in RPMI containing 10% heat inactivated fetal ovine serum (Hyclone Laboratories, Lagan, UK) supplemented with L-glutamine (4 mM) and gentamicin (5 μg/ml) (cMedium) in H_2O-saturated 5% CO_2 atmosphere at 37 °C. BV-2 microglia were tested periodically and resulted negative for mycoplasma contamination.

2.3. Transfection

BV-2 microglia were transfected with human RAGE cDNA or RAGEΔcyto cDNA (kindly provided by Dr. Henri J. Huttunen, Helsinki, Finland) using the plasmid vector pcDNA3 and the LipofectAMINE technique (Lipofectamine 2000, Life Technologies), according to maufacturer's instruction (Lipofectamine/DNA ratio=2:1). Briefly, 1×106 BV-2 cells, cultured in six-well plates and in 10% FBS medium without antibiotics, were transfected with 4-μg DNA (diluted in 200 μl OPTI-MEM Glutamax, Life Technologies) and 8-μg Lipofectamine Reagent (diluted in 200 μl OPTI-MEM Glutamax). Geneticin (G418) (375 μg/ml, Life Technologies) was added to the medium 48 h after transfection to select the transfected clones. Stable transfection was maintained by culturing the cells in media containing high levels of G418 (375 μg/ml). BV-2 microglial clones overexpressing RAGE or RAGEΔcyto (or mock transfected) were selected by limiting dilution and used in experiments as described above. Transient transfections were carried out using LipofectAMINE 2000 (Invitrogen) as recommended by the manufacturer. Briefly, cells cultured in 10% FBS without antibiotics were transfected with the expression plasmid MKK6AA (carrying an inactive mutant of MAP kinase kinase 6—MKK6), the NF-κB-luc reporter gene or the empty vector. After 24 h, cells were shifted to culture medium containing S100B. After further 18 h, cells were harvested to measure NO_2^- release, in the case of MKK6AA transfection, and luciferase activity, in the case of NF-κB transfection.

2.4. S100B treatment of BV-2 cells

Wild-type BV-2 cells, BV-2 cells stably transfected with either full-length RAGE or RAGEΔcyto and mock-transfected BV-2 cells (5×10^5) were seeded in 24-multiwell plates in the presence or absence of S100B alone or in combination with IFN-γ (50 U/ml). As positive controls, cells were treated with lipopolysaccharide (1 μg/ml) alone or in combination with IFN-γ (10 ng/ml+50 U/ml, respectively) (data not shown). In most experiments, cells were incubated for 18 h at 37 °C, 5% CO_2. Supernatants

were collected, spun down at 2000 rpm for 10 min, and tested immediately for NO_2^- release. Nitrite (NO_2^-) concentration in culture supernatants from S100B-treated microglial cells was used as a relative measure of NO release and was assayed by standard Griess reaction adapted to microplates [37]. Lower detection limit was equal to 3.125 nM of NO_2^-. In all experiments, parallel BV-2 cells treated as described above were used for cell counts. Thus, NO production was measured on a per cell basis. Each sample was tested in triplicate. Where appropriate, the following kinase inhibitors were used: 30 μM PD98059 (an inhibitor of the extracellular signal-regulated kinase [ERK1/2] upstream kinase, MAP kinase kinase [MEK]), 20 μM SB203580 (an inhibitor of p38 MAPK), 10 μM LY294002 (an inhibitor of the Akt upstream kinase, phosphatydilinositol-3-kinase [PI3-K]) (all from Calbiochem), and SP600125 (an inhibitor of JNK) (Alexis) at varying doses. In some experiments, the antioxidant N-acetylcysteine (Nac) (Zambon) or pyrrolidine carbodithioic acid (PDTC) (Sigma) at varying doses was used.

2.5. Kinase assay

ERK1/2, p38 MAPK and JNK assays were performed on BV-2 cell extracts subjected to SDS-PAGE and Western blotting using a monoclonal anti-phospho-ERK1/2 (Thr202/Tyr204) antibody (1:2000), a polyclonal anti-phospho-p38 MAPK (Thr180/Tyr182) antibody (1:1,000) and a polyclonal anti-phospho-JNK (Thr183/Tyr185) antibody (1:1,000), respectively (all from Cell Signal Technology). Total ERK1/2 in cell lysates was analyzed by a polyclonal anti-ERK1/2 (1:20,000) (Sigma). A monoclonal anti-α-tubulin was used to monitor protein loading on SDS gels. Peroxidase-conjugated secondary antibodies were from Sigma. Antibodies were diluted in blocking buffer (10 mM Tris–HCl, pH 7.4, 0.1 M NaCl, 5% nonfat dried milk powder, 0.1% Tween-20). To produce cell extracts, BV-2 cells were solubilized with 2.5% SDS, 10 mM Tris–HCl, pH 7.4, 0.1 M dithiothreitol, 0.1 mM TPCK protease inhibitor (Roche). The immune reaction was developed by enhanced chemiluminescence (ECL) (SuperSignal West Pico, Pierce).

2.6. Reverse transcriptase-PCR

Total cytoplasmic RNA was isolated from BV-2 microglia using the TRI-ZOL Reagent method. Subsequent steps were done as described [28]. The expected PCR product for rat RAGE was 453 bp. The expression of human RAGE and RAGEΔcyto was confirmed by reverse transcriptase (RT)-PCR using the following RAGE-specific oligonucleotides: (5′ to 3′)TGTCGG GATCCAGGATGAGG (forward primer) (250 nM) and (5′ to 3′)ACCACCAATTGGACCTCCTC (reverse primer 1) (250 nM) or (5′ to 3′)ACTACTCTCGCCTGCCTCAG (reverse primer 2) (250 nM). Reverse primer 2 was chosen for its specificity for a region contained in the RAGE C-terminal and transducing domain. The primers are specific for the human RAGE gene. The expected PCR products were 462 bp (RAGE Δ cyto) and 983 bp (RAGE full length) for the reverse primers 1 and 2, respectively. No PCR product was detected in control mock transfected BV-2 cells, containing only the murine RAGE gene. After amplification, samples (10 μl) of each PCR mixture were electrophoresed on a 1.3% agarose gel and RT-PCR products were revealed by ethidium bromide staining.

2.7. Statistical analysis

Each experiment was repeated at least three times. Representative experiments are depicted in the figures. Data were evaluated using the Student's *t* test.

3. Results

3.1. p38 MAPK and JNK mediate S100B-stimulated production of NO by BV-2 cells

In accordance with previous work [33], S100B stimulated NO production by BV-2 microglia in a dose-dependent manner in the presence of IFN-γ (Fig. 1). Inhibition of the MEK-ERK1/2 pathway by PD98059 resulted in a remarkable reduction of IFN-γ-dependent, but not S100B-dependent, NO production (Fig. 1). In contrast, inhibition of p38 MAPK by SB203580 resulted in a strong reduction of both IFN-γ- and S100B-dependent NO production (Fig. 1). Under the present experimental conditions, neither PD98059 nor SB203580 treatment caused changes in BV-2 cell numbers, as assayed by the MTT method (data not shown). Collectively, these data suggested that S100B stimulated NO

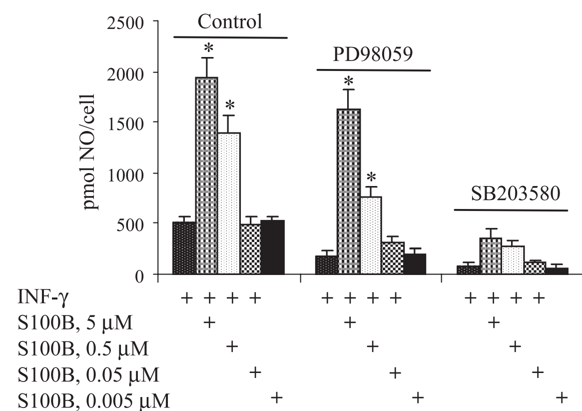

Fig. 1. S100B-stimulated NO production by BV-2 microglia is dependent on p38 MAPK activity and independent of ERK1/2 activity. BV-2 cells were exposed to IFN-γ in the presence of increasing amounts of S100B plus or minus the MEK inhibitor, PD98059, or the p38 MAPK inhibitor, SB203580. Results are expressed as pmol NO/cell. Average of three determinations±S.D. *Significantly different from control (*P*<0.01).

production via activation of p38 MAPK, but not MEK-ERK1/2. Western blot analyses documented that relatively high doses of S100B could activate ERK1/2 to some extent in the presence, but not in the absence, of IFN-γ (Fig. 2A). However, S100B-induced activation of ERK1/2 was unrelated to S100B-dependent increase in NO production, as shown in Fig. 1. In contrast, S100B activated p38 MAPK dose-dependently in the presence of IFN-γ (Fig. 2B), which correlated positively with the strong reduction of IFN-γ/S100B-dependent NO production in the presence of SB203580. Also, relatively high doses of S100B activated p38 MAPK in the absence of IFN-γ (Fig. 2B), but this event was unrelated to NO production since under these conditions S100B was unable to elicit any NO response in BV-2 cells (Ref. [33]; also see Fig. 3). Incidentally, these data suggested that S100B might elicit additional responses in BV-2 microglia independently of IFN-γ. Interrelation between S100B-dependent activation of p38 MAPK and S100B-induced stimulation of NO production in the presence of IFN-γ was also documented by the observation that SB203580 caused a strong reduction of IFN-γ/S100B-induced phosphorylation of p38 MAPK, while no such reduction was seen following treatment with the MEK-ERK1/2 inhibitor, PD98059, or LY294002, an inhibitor of the Akt (PKB) upstream kinase, PI3-K (Fig. 2C). Further evidence that S100B-induced stimulation of NO production by BV-2 microglia was dependent on p38 MAPK activation came from experiments performed using cells that had been transiently transfected with MAKK6AA, an inactive mutant of the p38 MAPK upstream kinase, MKK6. In this case, virtually no S100B-dependent NO production could be observed (Fig. 3).

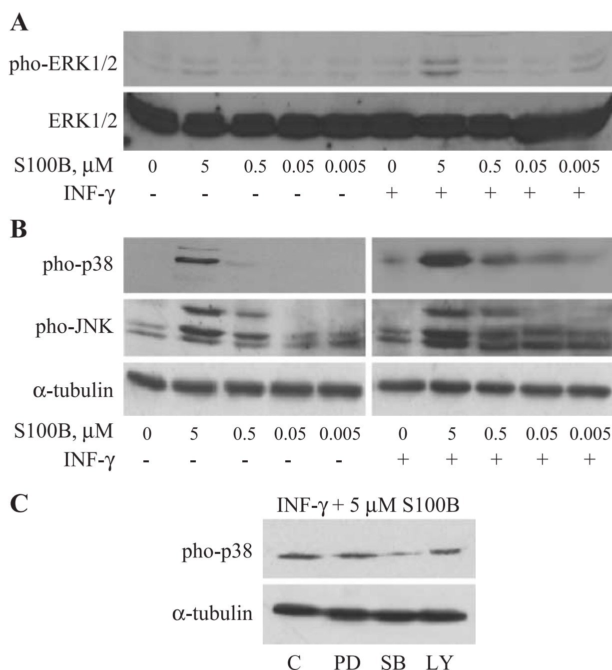

Fig. 2. S100B activates p38 MAPK and JNK in BV-2 microglia. Conditions were as described in Fig. 1 except that cells were lysed and extracts were subjected to Western blotting using an anti-phospho-ERK1/2 (A), an anti-phospho-p38 MAPK (B) or an anti-phospho-JNK antibody (B). Shown in C is a Western blotting analysis of phospho-p38 MAPK in BV-2 microglia exposed to IFN-γ plus S100B in the absence or presence of PD98059, SB203580 or LY294002. Representative of three experiments.

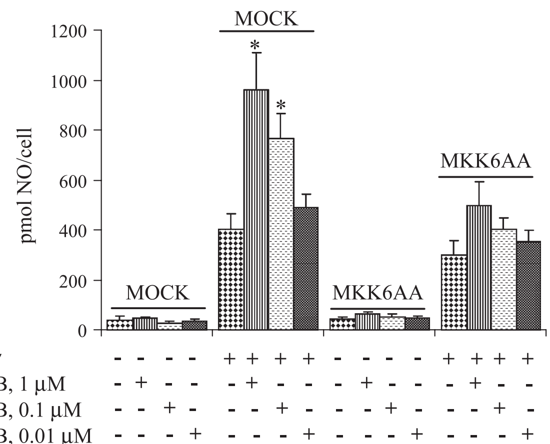

Fig. 3. Transient transfection of BV-2 microglia with MKK6AA reduces S100B-stimulated NO production. Conditions were as described in Fig. 1 except that cells were transiently transfected with empty vector (mock) or expression plasmid MKK6AA (an inactive mutant of the p38 MAPK upstream kinase, MKK6). Results are expressed as pmol NO/cell. Average of three determinations±S.D. *Significantly different from control (*P*<0.01).

S100B also caused a dose-dependent activation of JNK, another stress-activated kinase, both in the absence and presence of IFN-γ, although to a slightly larger extent in the presence of IFN-γ (Fig. 2B). Consistently, inhibition of JNK by treatment with SP600125 resulted in the inhibition of S100B-dependent stimulation of NO production (Fig. 4). However, since S100B caused NO production only in the presence of IFN-γ (Ref. [33]; also see Fig. 3), S100B-induced

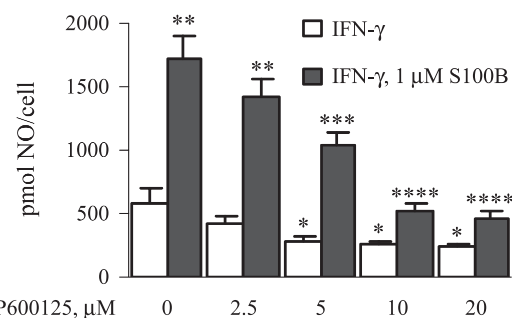

Fig. 4. S100B-stimulated NO production by BV-2 microglia is dependent on JNK activity. Conditions were as described in Fig. 1 except that BV-2 microglia were exposed to increasing doses of the JNK inhibitor, SP600125. Results are expressed as pmol NO/cell. Average of three determinations±S.D. *Significantly different from control (no inhibitor present) (*P*<0.01). **Significantly different from internal control and from the IFN-γ alone/no inhibitor condition (*P*<0.01). ***Significantly different from internal control, the IFN-γ alone/no inhibitor condition and the IFN-γ-S100B/no inhibitor condition (*P*<0.01). ****Significantly different from internal control and the IFN-γ-S100B/no inhibitor condition (*P*<0.01).

activation of JNK in the absence of IFN-γ might serve an additional function.

3.2. S100B-stimulated production of NO by BV-2 microglia is independent of RAGE signaling

S100B has been shown to signal via interaction with RAGE in a number of cell types [16,18,24], although RAGE-independent effects of S100B have been reported [28,29], indicating that RAGE might not be the sole S100B receptor. RAGE was originally detected in monocytes/macrophages and BV-2 cells, and S100B was shown to activate microglial RAGE [24]. As both p38 MAPK and JNK can be activated by RAGE engagement [19,21,23], we reasoned that RAGE might transduce the effect of S100B on NO production in BV-2 microglia. Thus, we generated BV-2 cell lines stably overexpressing either RAGEΔcyto, a RAGE mutant lacking the cytoplasmic and transducing domain (BV-2/RAGEΔcyto microglia), or full-length RAGE (BV-2/RAGE microglia). Overexpression of RAGEΔcyto and full-length RAGE in BV-2 microglia was documented by RT-PCR (Fig. 5). To our surprise, we found that not only was S100B able to stimulate NO production in BV-2/RAGEΔcyto microglia, but that the extent of stimulation by S100B in these cells was significantly greater than that in BV-2/mock microglia (Fig. 6). Also, similar extents of S100B-dependent stimulation of NO production were detected in BV-2/RAGEΔcyto and BV-2/RAGE microglia (Fig. 6). Thus, the S100B effect on NO production in BV-2 microglia was independent of RAGE signaling; however, the higher density of RAGE molecules on BV-2 cell surface resulted in a larger extent of S100B-induced NO production. This suggested the possibility that S100B might bind to RAGE and concentrate on the BV-2 cell surface via RAGE binding, thereby triggering NO production by a mechanism other than RAGE signaling.

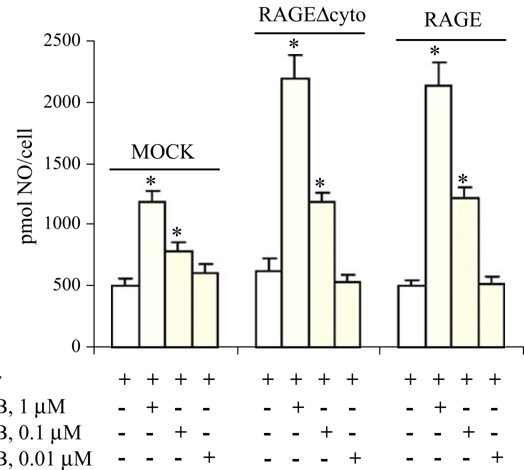

Fig. 6. BV-2/RAGEΔcyto microglia and BV-2/RAGE microglia show increased NO production in the presence of S100B. BV-2/mock, BV-2/RAGEΔcyto and BV-2/RAGE microglia were exposed to increasing doses of S100B in the presence of IFN-γ. Results are expressed as pmol NO/cell. Average of three determinations±S.D. *Significantly different from control (IFN-γ/no S100B present, BV-2/mock microglia) and internal control ($P<0.01$).

3.3. S100B-induced increase in NO production in BV-2 microglia depends on ROS production

The antioxidant agent Nac strongly reduced the amount of NO produced by BV-2 microglia exposed to IFN-γ and to IFN-γ/S100B, irrespective of the BV-2 clone used (i.e., BV-2/mock, BV-2/RAGEΔcyto, BV-2/RAGE) (Fig. 7). Thus, both IFN-γ- and IFN-γ/S100B-dependent NO production relied on stimulation of ROS production. However, IFN-γ

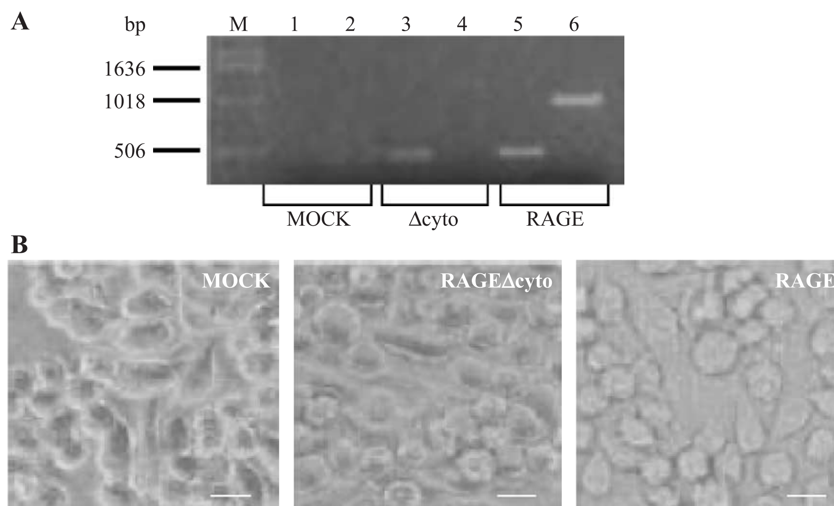

Fig. 5. Characterization of BV-2 microglia transfected with full-length human RAGE or human RAGEΔcyto. (A) Shown are PCR products obtained with human RAGE reverse primer 1 (lanes 1, 3 and 5) and reverse primer 2 (lanes 2, 4 and 6). No PCR products could be obtained with reverse primer 2 in the case of RAGEΔcyto-transfected BV-2 microglia (lane 4) due to specificity of this primer for a region present in full-length RAGE and absent from RAGEΔcyto. No PCR products could be obtained with either primer in the case of mock-transfected BV-2 microglia (lanes 1 and 2). (B) Transfection with empty vector, human RAGEΔcyto or full-length human RAGE does not change the BV-2 phenotypic characteristics. Bars=5 μm.

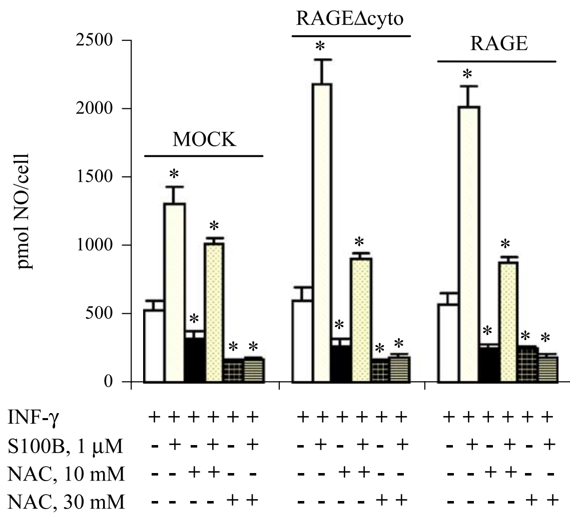

Fig. 7. S100B-stimulated NO production by BV-2 microglia depends on ROS production. Conditions were as described in Fig. 6 except that BV-2 microglia were exposed to increasing doses of Nac. Results are expressed as pmol NO/cell. Average of three determinations±S.D. *Significantly different from control (IFN-γ/no S100B present, BV-2/mock microglia) and internal control ($P<0.01$).

and IFN-γ/S100B displayed different Nac requirements, in that higher doses of Nac were required to block IFN-γ/S100B-induced NO production than to block IFN-γ-induced NO production (Fig. 7) likely due to the synergistic effect of IFN-γ and S100B.

3.4. S100B activates NF-κB transcriptional activity in BV-2 microglia in a RAGE-mediated manner

RAGE engagement by S100B in BV-2 microglia [24] and neurons [16] was shown to cause activation of the transcription factor NF-κB, and NF-κB activation is known to be associated to iNOS induction and NO production [38]. Also, as mentioned earlier, S100B was shown to up-regulate iNOS expression in the presence of IFN-γ [33]. To obtain information about the role of NF-κB in S100B-mediated NO production, BV-2/RAGE, BV-2/RAGEΔcyto and BV-2/mock microglia were treated with PDTC, a sulfhydryl antioxidant with the ability to inhibit NF-κB function. As observed with Nac treatment (Fig. 7), PDTC strongly reduced the amount of NO produced by BV-2 microglia exposed to IFN-γ both in the absence and in the presence of S100B, irrespective of the BV-2 clone used (Fig. 8); however, as observed in Nac experiments shown in Fig. 7, IFN-γ and IFN-γ/S100B displayed different PDTC requirements, in that higher doses of PDTC were required to block IFN-γ/S100B-induced NO production than to block IFN-γ-induced NO production (Fig. 8). These data suggested that the inhibitory effect of PDTC treatment on S100B-induced NO production by BV-2 cells might depend on the antioxidant properties of the chemical a larger extent. To verify this possibility, BV-2/mock, BV-2/RAGEΔcyto, BV-2/RAGE microglia were analyzed for activation of NF-κB transcriptional activity by a luciferase assay (Fig. 9). We found that: (1) S100B stimulated NF-κB transcriptional activity in BV-2/mock microglia in the absence of cofactors; (2) extremely low, if any, levels of NF-κB activation were measured in BV-2/RAGEΔcyto microglia, with no changes induced by S100B; and (3) a robust increase in NF-κB activation was seen in BV-2/RAGE microglia, which was remarkably increased by S100B. Thus, differently from the effects of S100B on NO production, the ability of S100B to stimulate NF-κB transcriptional activity in BV-2 microglia was strictly dependent on RAGE transducing activity.

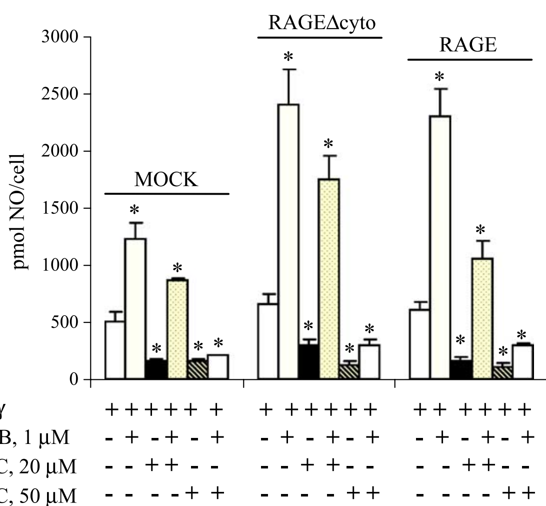

Fig. 8. S100B-stimulated NO production by BV-2 microglia is largely independent of NF-κB activation. Conditions were as described in Fig. 7 except that BV-2 microglia were exposed to increasing doses of PDTC. Results are expressed as pmol NO/cell. Average of three determinations±S.D. *Significantly different from control (IFN-γ/no S100B present, BV-2/mock microglia) and internal control ($P<0.01$).

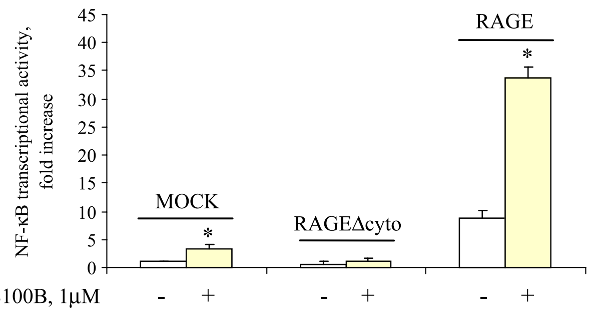

Fig. 9. S100B activates NF-κB transcriptional activity independently of IFN-γ, in a RAGE-mediated manner. BV-2/mock, BV-2/RAGEΔcyto and BV-2/RAGE microglia were transiently transfected with NF-κB-luc reporter gene and then incubated in the absence or presence of S100B. Results are expressed as fold increase in NF-κB transcriptional activity.

4. Discussion

While brain extracellular S100B seems to be primarily involved in trophic activities towards neurons under normal conditions [1,2,27], increases in its concentration in the brain extracellular space might be detrimental to neurons in

several manners, including stimulation of NO production by both astrocytes and microglia [1,2,27]. This latter effect, however, depends on the simultaneous presence of factors such as lipid A or IFN-γ [32,33], suggesting that high levels of S100B might sensitize astrocytes and microglia to bacterial lipid products and pro-inflammatory cytokines. In this scenario, either the presence of high levels of extracellular S100B (as observed in Down's syndrome, Alzheimer's disease, brain infectious diseases and epilepsy [1,2,27]) might amplify the inflammatory response in the brain, or vascular and/or infectious insults might result in an increased release of the protein. While this latter event might represent an attempt to protect neurons against noxious stimuli during the initial stages of the insult, it might change into a deleterious one in case of excessive levels attained by extracellular S100B. S100B has been shown to interact with RAGE in microglia and neurons [16,24], and RAGE has been reported to transduce both trophic and toxic effects of S100B on neurons and regulatory effects of the protein on microglia [16,24], while uncertainty remains as to the receptor transducing the S100B's effects on astrocytes [1,2,27].

Aside from the original work describing the ability of S100B to interact with RAGE in microglia thereby causing activation of NF-κB [24], little information is available about the mechanism by which the protein sensitizes microglia to microglia-activating factors. The present data show that at relatively high and potentially detrimental doses, S100B causes stimulation of NO production in BV-2 microglia cells in the presence of IFN-γ via production of ROS and activation of the stress-activated kinases, p38 MAPK and JNK. However, these effects are independent of RAGE signaling and NF-κB activation to a large extent, though dependent on the density of RAGE molecules expressed on the microglial cell surface. Several lines of evidence support this conclusion. First, the p38 MAPK inhibitor, SB203580, and the JNK inhibitor, SP600125, each reduce S100B-stimulated NO production by BV-2 microglia, and transfection of BV-2 cells with MKK6AA, an inactive mutant of the p38 MAPK upstream kinase MKK6, results in a reduction of S100B-stimulated NO production. Second, the antioxidant Nac also reduces S100B-stimulated NO production, suggesting an important role of ROS in this S100B effect. Third, S100B is still capable of stimulating NO production by BV-2 microglia stably transfected with RAGEΔcyto, a signaling incompetent mutant of RAGE, and the amount of NO produced on a per cell basis under these conditions is larger than that produced by mock-transfected cells but similar to that produced by full-length RAGE-transfected cells. Thus, S100B seems to stimulate NO production independently of RAGE signaling, but dependently on the density of RAGE extracellular domain. Lastly, while S100B stimulates NF-κB transcriptional activity in a RAGE-mediated manner in BV-2 microglia in the absence of IFN-γ (i.e., no such effect can be seen in BV-2/RAGEΔcyto microglia, and BV-2/RAGE microglia exhibit a 10-fold increase in NF-κB transcriptional activity compared with BV-2/mock microglia), no relationship can be seen between S100B-stimulated NO production in BV-2 microglia and NF-κB transcriptional activity, since S100B does not require the presence of cofactors to cause activation of NF-κB transcriptional activity while it necessitates the presence of IFN-γ to stimulate NO production. Moreover, the percent increase in S100B-dependent augmentation of NO production in BV-2/RAGE and BV-2/RAGEΔcyto microglia compared with BV-2/mock microglia does not correlate with the manifold increase in NF-κB transcriptional activity caused by S100B in BV-2/RAGE microglia compared with BV-2/mock microglia. However, the observation that S100B stimulates NF-κB transcriptional activity in a RAGE-mediated manner in BV-2 microglia indicates that the protein does activate RAGE transducing activity. In this respect, RAGE-mediated increase of ROS production and NF-κB activation by S100B in neurons have been reported [16]. Thus, we cannot exclude that part of the S100B-stimulated NO production in BV-2 microglia might depend on RAGE engagement and activation. In line with this is the observation that at relatively low doses the antioxidant NF-κB inhibitor, PDTC, causes ~30% decrease in IFN-γ/S100B-induced NO production in BV-2/mock and BV-2/RAGEΔcyto microglia and ~60% decrease in BV-2/RAGE microglia, which suggests that NF-κB activation might be in some way functionally linked to RAGE engagement and activation by S100B in the process of S100B-stimulated production of NO in microglia, in part. Precedent exists for S100B's ability to stimulate NO production in astrocytes via NF-κB activation [39]. However, the RAGE-mediated ability of S100B to stimulate NF-κB transcriptional activity in microglia likely is linked to other and independent effects of the protein. Likewise, as S100B is capable of activating both p38 MAPK and JNK independently of IFN-γ without causing NO production, our data clearly indicate that effects of S100B on microglia are not restricted to NO production. Future analyses should identify the additional effects caused by S100B interaction with RAGE in microglia.

The finding that S100B does not use the RAGE transducing activity to synergize with IFN-γ in the stimulation of NO production but it does use RAGE extracellular domain is intriguing. We hypothesize that binding of S100B to RAGE in microglia might serve the function of concentrating the protein at the cell surface thereby permitting S100B either to activate a co-receptor or to cause itself increased production of ROS by an unknown mechanism, activation of p38 MAPK and JNK and stimulation of NO production. An alternative possibility is that the increased density of RAGE extracellular domain in BV-2/RAGEΔcyto microglia might result in an increased activation of endogenous RAGE by means of receptor

clustering. However, this is unlikely because in this case one would expect to measure much larger amounts of NO produced by BV-2/RAGE microglia compared to BV-2/RAGEΔcyto microglia than actually measured.

In conclusion, the present data support a role of high levels of S100B in microglia activation, which could be important in the pathophysiology of neurodegenerative processes.

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