INTRODUCTION

One of the features of asthma is persistent pulmonary inflammation with increased numbers of activated T-lymphocytes, eosinophils and macrophages/monocytes, and resident tissue cells. These cells play a role in establishment and maintenance of the chronic inflammatory process by releasing a wide repertoire of cytokines, chemokines, and other proinflammatory mediators. There is increasing evidence that reactive oxygen and nitrogen species are also important mediators of the inflammatory process, providing a cellular mechanism that links oxidative stress and chronic inflammation. Oxidants are a well-characterized source of cellular injury at multiple levels, and they are believed to produce many of the features typical of asthma (Cararo and Papi, 2004). On the other hand, as intracellular molecular messengers, they regulate specific and redox-dependent signaling pathways modulating the expression of a variety of genes, among which are the genes coding for cytokines (Morel and Barouki, 1999).

Binding of a ligand to the glucocorticoid receptor (GR) is an important step in regulation and modulation of glucocorticoid signal transduction. Upon interaction with a steroid, the GR becomes activated to a form that can interact with specific regulatory DNA elements in the promoter region of glucocorticoid-sensitive genes, thereby modifying their transcription. Alternatively, the activated receptor can exert its transactivation or transrepression activity by interacting with other regulatory proteins, thus mediating most of the anti-inflammatory and immunosuppressive actions of glucocorticoid hormones. Extensive studies have shown that the GR is subject to redox modulation in vitro.
(Silva and Cidlowski, 1989; Meshinchi et al., 1990), as well as in vivo (Makino et al., 1996; Galigniana et al., 1999). By virtue of being a redox-sensitive transcription factor, the Gr is a potential target through which reactive species can modulate the expression of glucocorticoid-dependent genes especially in asthma, where reactive oxygen species are important factors of the disease pathogenesis (Carabini and Pap, 2004). Additionally, oxidative stress and proinflammatory mediators can enhance the inflammatory response and modulate corticosteroid function by repression of HDAC2 activity (Ito et al., 2004). Attenuation of this activity was found to be associated with nitration of the enzyme’s tyrosine residues, which can be provoked by H$_2$O$_2$ or peroxynitrite.

In this study, we performed initial experiments to test a hypothesis that the pathogenesis and/or maintenance of the chronic disease in asthmatic patients can be attributed to defects in Gr function possibly resulting from the action of oxidants. To that end, we conducted a series of ex vivo treatments of peripheral lymphocytes with oxidizing agents and compared resulting Gr functional alterations with the alterations previously registered in asthmatic patients with mild and moderate asthma (Perišić et al., 2008).

MATERIALS AND METHODS

Blood sampling and cell isolation

Blood samples were obtained from healthy adult volunteers by venipuncture. Blood was collected in heparinized tubes (25 U/ml), and peripheral blood mononuclear cells (PBMCs) were isolated within one hour from blood drawing. To isolate the cells, blood was diluted with an equal volume of phosphate-buffered saline (PBS) (0.14 mmol/l NaCl, 2.7 mmol/l KCl, 1.5 mmol/l KH$_2$PO$_4$, 6.5 mmol/l Na$_2$HPO$_4$; pH 7.2) and subjected to density gradient centrifugation over Ficoll-Paque Plus (Amersham Biosciences) at 380 x g for 30 min at 23°C. The cells were thoroughly washed with PBS and resuspended in RPMI-1640 medium (Gibco/Invitrogen). Cell viability, evaluated by the Trypan Blue dye exclusion method, was always found to be greater than 95%. The PBMCs that were not used immediately were rapidly frozen in liquid nitrogen.

Hormone binding assay

The cell suspension was adjusted to a final density of 5x10$^6$ cells/ml. Aliquots of mononuclear cells, containing 1x10$^6$ cells each, were incubated with $[^3]$H)dexamethasone (Amersham Biosciences, spec. act. 34 to 40 Ci/mmol) at five or six different concentrations (depending on PBMC yield) ranging from 5 to 120 nM in triplicate in the absence or presence of a 100-fold molar excess of unlabeled dexamethasone (Sigma Chemical Co., MO, USA) to determine total and nonspecific binding, respectively. After 2 h of incubation in a water bath at 37°C, all tubes were placed on ice and the reaction stopped by adding 1 ml of ice-cold PBS. Following three cycles of washing at 4°C with PBS and centrifugation at 1000 x g for 5 min at 4°C, the final pellets were resuspended in PBS and transferred to 3 ml of a scintillation cocktail (Ecoscnt H, National Diagnostic Inc). Radioactivity measurements were performed in a Rackbeta scintillation counter (LKB) with approximately 50% efficiency for tritium. Specific binding was calculated by subtracting the nonspecific from total binding at each dexamethasone concentration. To assess inter-assay variation, repeated measurements were performed on different occasions. For determination of the GR number, an intra-individual variation of 18% was registered, while the coefficient of variation for its hormone-binding affinity was found to be 22%, these data being in agreement with existing reports (Hearing et al., 1999; Hagendorf et al., 2005). The number of receptor sites per cell and apparent dissociation constant (K$_d$) were calculated using computer-assisted nonlinear regression curve fitting procedure and assuming competitive binding to one class of binding sites.

Treatment of PBMCs with oxidizing agents

Mononuclear cells were isolated and resuspended in RPMI-1640 medium containing 2 mM glutamine, 25 mM HEPES, and 10% heat-inactivated fetal bovine serum (FBS).
The cells were incubated in the presence of 1 mM 3-morpholinosydnonimine hydrochloride (SIN-1, Sigma Chemical Co), 1 mM S-nitroso-N-acetylpenicillamine (SNAP, Sigma Chemical Co.), or 1 mM H₂O₂ (Sigma Chemical Co.) for 6 h at 37°C. The first of these agents (SIN-1) releases superoxide anion (O₂⁻) and nitrogen monoxide (NO) in equimolar quantities, thereby producing peroxynitrite anion (ONO₂⁻), whereas SNAP is a pure NO donor. Treatment with SIN-1 was performed according to Martin-Romero et al. (2004). Briefly, in order to obtain a steady-state concentration of short-lived ONO₂⁻ during 6 h, we applied sequential SIN-1 pulses to simulate chronic exposure. The initial 90 mM stock solution of SIN-1 was freshly prepared in acidic buffer (pH 5.0) and kept on ice in the dark.

After the treatment, the medium was replaced by fresh medium and cell viability was estimated. An aliquot of the mononuclear cell suspension from each treatment was stored in liquid nitrogen for subsequent analysis. The rest was subjected to radioligand-binding assay and analyzed for GR binding parameters.

**Cellular protein extraction**

For protein extraction, cellular pellets (1×10⁶ cells) were resuspended in 15 μl of ice cold hypotonic lysis buffer (50 mmol/l NaCl, 5% glycerol, 0.5% Triton X-100, 2 mmol/l ethylenediaminetetraacetic acid, and 50 mmol/l Tris; pH 7.5) supplemented with a complete protease inhibitor cocktail (Roche Diagnostics GmbH). Following incubation on ice for 15 min and two freeze/thaw cycles, the lyzate was centrifuged at 13,000 x g for 15 min at 4°C. The supernatants, referred to as whole cell extracts, were stored in small portions at -75°C. Protein concentration ranged from 3 to 6 μg/μl.

**Western blotting**

The proteins were reduced and denatured by 5-min treatment at 95°C with a buffer containing 62.5 μM Tris (pH 6.8), 2% SDS, 10% glycerol, 5% β-mercaptoethanol, and 0.0075% bromphenol blue. The proteins (70 μg per lane) were resolved on 7.5% SDS-PA gel, together with protein molecular mass standards (29-205 kDa, Sigma Chemical Co.). After overnight transfer, PVDF membranes (Hybond-P, Amersham Biosciences) were incubated in a blocking buffer containing 2.5% nonfat dry milk in PBS (pH 7.2) for 1.5 h at room temperature. The GR was detected using two different anti-GR polyclonal antibodies: PA1-511A (1:1000 dilution; Affinity Bioreagents, Golden, CO) and GR M-20 (1:500 dilution; Santa Cruz Biotechnology, Santa Cruz, CA). The former antibody, herein designated Ab1, was raised against an immunizing peptide corresponding to amino acid residues 346-367 of the human GR (Cidlowski et al., 1990). The latter antibody, designated Ab2, was raised against N-terminal amino acids of murine GR. After three cycles of washing with PBS containing 0.1% Tween 20, the membranes were incubated for 1 h with alkaline phosphatase-conjugated secondary antibody. The immunoreactive protein bands were visualized on STORM (Amersham Pharmacia Biotech UK Limited, England) by an enhanced chemifluorescent method (ECF, Amersham Biosciences). The membranes were reprobed with mouse monoclonal anti-β-actin antibody (1:8000 dilution; Sigma Chemical Co.) to verify equal protein load in each lane. Since protein nitration was used as a measure of ONO₂⁻ formation, monoclonal anti-nitrotyrosine antibody (1:500; Upstate Biotechnology Inc., Lake Placid, NY) was also used. For positive control, BSA at 10 mg/ml in PBS (pH 7.4) was incubated with 10 mM SIN-1 for 2 h at 37°C. Relative integrated optical density of immunoreactive bands was measured using Image Quant software (Amersham Biosciences Limited, UK) and the results expressed in arbitrary units (AU).

**Statistical analyses**

The relative levels of GR protein and its functional parameters before and after the treatments were compared by the nonparametric Kruskal-Wallis test, followed by an appropriate post hoc test. Between-group differences were analyzed with the Mann-Whitney U-test, or with the t-test, where appropriate. A two-tailed p value of less than 0.05 was considered significant. Statistical comparisons were carried out using GraphPad Prism, version 4.0 (GraphPad Prism Software Inc., San Diego, CA).
RESULTS

To examine the effects of several different oxidizing agents on GR ligand–binding parameters in PBMCs, a set of experiments was performed using freshly isolated PBMCs obtained from the blood of healthy adult donors. Pilot experiments were carried out to determine optimal conditions for cell treatment that produced no effect on viability of the cells, as measured by the Trypan Blue exclusion method. Cells were treated with 1 mM SIN-1, 1 mM SNAP, and 1 mM H₂O₂ and subsequently assayed for [³H]dexamethasone binding. Figure 1A shows [³H]dexamethasone binding curves before and after treatment of PBMCs with oxidizing agents. The equilibrium dissociation constant (k_d) of the hormone-receptor interaction, calculated from the binding curves by computer-assisted analysis, was increased by treatment with SIN-1 (57.3 ± 5.9 nM; p=0.003) and H₂O₂ (82.3 ± 10.0 nM; p=0.003), but remained unchanged after treatment with SNAP (12.6 ± 1.3 nM) as compared to k_d in untreated PBMCs (10.3 ± 2.4 nM) (Fig. 1B). On the premise that k_d is inversely proportional to the receptor’s affinity for the hormone, the results demonstrate that treatment with SIN-1 and H₂O₂ led to a decrease of the receptor’s ligand-binding affinity, while SNAP left it unchanged. Furthermore, after treatment with SIN-1 the number of dexamethasone-binding sites per cell was severely decreased (606 ± 127; p=0.007) in comparison with untreated cells (4125 ± 852). In contrast, treatment with H₂O₂ caused an elevation in the number of receptor sites in PBMCs (12556 ± 1174; p=0.006), whereas treatment with SNAP did not affect it (4010 ± 511; n=2) (Fig. 1C).

When Western blot analysis of GR bands was done using Ab1 antibody, a specific pattern of the GR bands was obtained both before and after treatment with oxidants. In untreated cells, in addition to a rather weak 94-kDa band, corresponding to the full-length GR, immunospecific bands migrating at approximately 60, 55, 50, 45, and 29 kDa, corresponding to truncated GR molecules, were also detected (Fig. 2A, upper left panel). A similar pattern of immunoreactive bands was obtained with PBMCs of another blood donor (Fig. 2A, upper right panel). Treatment with SIN-1 reduced the intensity of all immunoreactive protein bands, especially those with lower molecular masses. The pattern obtained after treatment with SNAP resembled that found in PBMC extracts after treatment with SIN-1. On the
other hand, H₂O₂ treatment yielded a prominent band migrating at around 60 kDa, while reactivity of the 50- and 29-kDa bands was comparable to that in untreated cells. After the blots were reprobed with Ab2, the 94 kDa band became much more pronounced in all samples (Fig. 2A, middle panels), while the lower-molecular-mass isoforms were not detected. Analysis of GR/actin ratios revealed that SIN-1 and H₂O₂ treatments produced a slight decrease and an increase, respectively in intensity of the 94-kDa band, although these differences were not statistically significant (Fig. 2B).

In principle, changes of GR binding activity such as those registered in our study upon treatment of PBMC extracts with SIN-1, that is decrease
in both GR number and hormone-binding affinity, could be a consequence of the receptor's covalent modification. Since SIN-1 is known to release NO and $\text{O}_2^-$, which react to produce a powerful oxidant and nitrating agent, ONO$_2^-$, we used an antibody raised against the protein nitrotyrosine to check for nitrination of proteins, specifically the GR, upon treatment with SIN-1. However, in our experiment no nitrated proteins in SIN-1-treated cellular extracts of PBMCs were detected, while BSA, used as a positive control, was successfully nitrated by the same treatment (Fig. 3).

**DISCUSSION**

In the present study, treatment of PBMCs of healthy adult blood donors with $\text{H}_2\text{O}_2$ was found to induce alterations of GR binding parameters, including decrease in the receptor's affinity for the ligand and increase in the number of receptor sites per cell. These alterations were similar to those observed in PBMCs isolated from the blood of pediatric patients suffering from moderate asthma, which in that respect differed from mild asthmatics and healthy controls (Perišić et al., 2008). Moreover, the pattern of GR protein bands obtained after treatment of PBMCs with $\text{H}_2\text{O}_2$ also resembled that observed in PBMCs of moderate asthmatics (Perišić et al., unpublished data). The similarity between the GR alterations produced by ex vivo treatment of PBMCs with $\text{H}_2\text{O}_2$ and those observed in PBMCs of moderate asthmatics suggests that pediatric moderate asthma might be associated with structural and functional modifications of the GR that are likely to result from $\text{H}_2\text{O}_2$-mediated oxidative stress.

There is considerable evidence that some post-translational modifications of the receptor can influence its ability to bind hormone. Oxidizing conditions such as those created by $\text{H}_2\text{O}_2$ treatment were previously shown to promote formation of intramolecular disulfide bonds, which alter the structure (Silva and Cidlowski, 1989; Meshinchi et al., 1990) and/or reversibly inactivate steroid-binding activity (Meshinchi et al., 1990; Makino et al., 1996) of the receptor. In this study, we found that treatment with $\text{H}_2\text{O}_2$ can induce a considerable decrease in ligand-binding affinity (i.e., increase of $K_d$), accompanied by increase in both the number of hormone-binding sites and levels of the GR protein in PBMCs from healthy donors. On the other hand, SNAP, as a NO donor, did not provoke any changes of GR function, whereas SIN-1, being a ONO$_2^-$ donor, dramatically reduced both GR binding parameters. The differences in effects of the oxidizing agents applied can probably be ascribed to different chemical modifications of the receptor. In contrast to studies presenting evidence for distinct effects of NO-donating compounds on GR affinity for the hormone in vivo (Galigniana et al., 1999; Paul-Clark et al., 2003), influence of the ONO$_2^-$ donor SIN-1 on GR binding properties has not yet been investigated. It has been experimentally confirmed and generally accepted that

![Fig. 1](image_url). Western blot analysis of nitrotyrosine modified proteins in cellular extracts of mononuclear cells. Bovine serum albumin treated with 10 mM SIN 1 for 2 h at 37°C in PBS, pH 7.4, was used as a positive control (lane 1). Nitrotyrosine was not detected in untreated PMBCs of healthy adult donors (lane 3) or cells treated with 1 mM SIN 1 for 6 h at 37°C (lane 4). The positions of molecular mass reference proteins are shown in lane 2.
both in oxygenated solution and in physiological conditions, SIN-1 releases NO and O₂ −. The product of their simultaneous reaction is ONO₂ −, a potent oxidizing agent that has been suggested to play a role in several pathological conditions, including asthma (Sal et al., 1998). Although the formation of nitrotyrosine is considered to be an indicator of ONO₂ − presence and activity, in our study anti-nitrotyrosine antibody failed to recognize corresponding epitopes in cellular extracts of PBMCs treated with SIN-1. This result suggests that the herein observed decrease in GR number and its hormone-binding affinity was not a consequence of GR nitration, or that these changes were indirectly induced by SIN-1 treatment. Additionally, it is also possible that the level of GR nitration was too low to be detected by immunoblotting, or that intracellular conditions hampered ONO₂ − formation, prompting SIN-1 to behave more like a NO donor (Singh et al., 1999). Thus, the precise mechanisms governing the observed functional modifications and changes of GR expression in mononuclear cells, including delineation between direct and indirect roles of oxidants, remain to be elucidated.

In regard to the role of inflammatory mediators in asthma pathophysiology and in glucocorticoid resistance, elevated GR number and diminished GR binding affinity have been described in asthmatic patients (Sher et al., 1994), as well as in PBMCs from healthy donors after treatment with IL-2 and IL-4 (Kam et al., 1993). The underlying mechanism of alterations in GR binding parameters has not yet been described. However, an increasing number of studies indicate that cytokines initiate the production of reactive oxygen and nitrogen species in various cell types (Ohba et al., 1994; Bohler et al., 2000). This cross-talk between cytokines and reactive species supports the assumption that a reactive species-mediated mechanism might be associated with the changes in GR binding characteristics observed after treatment with cytokines.

In our study, the GR level in PBMCs was also examined by Western blot analysis. The observed SIN-1- and H₂O₂-induced changes in the GR protein level, measured by Western blot, were parallel to those in the number of dexamethasone-binding sites determined by radioligand binding assay. Being based on the use of antibodies that recognize epitopes in denatured polypeptides, the Western blot method provided the means to detect GR protein regardless of its hormone-binding activity. In contrast, only receptor molecules that are unoccupied by the hormone can be detected by radioligand binding assay. Thus, immunoblotting is a way to assess the GR protein level that results from the balance between receptor synthesis and degradation, whereas binding studies disclose only functional receptor molecules that are able to bind ligand. Any discrepancy between results of the two methods suggests that the mechanisms regulating the receptor’s binding activity are implicated. In our study, however, treatment of mononuclear cells with oxidative agents produced changes in the number of functional GR molecules, which can be explained by similar changes in the GR protein level, suggesting that the agents applied did not affect regulation of GR binding activity. This suggestion seemingly contradicts our finding that H₂O₂ provoked a significant decrease in the receptor’s affinity for the hormone, as well as the well documented fact that H₂O₂ can inactivate steroid-binding activity of the GR, promoting formation of intramolecular disulfide bonds (Silva and Cidlowski, 1989; Meshinchi et al., 1990). However, inasmuch as treatment of PBMCs with H₂O₂ was herein shown to lead to a considerable increase in the receptor protein level, it can be assumed that this increase is large enough to overcome the peroxide-induced diminishment of the receptor’s hormone-binding activity.

It is well known that a cell’s sensitivity to glucocorticoids is directly determined by the level and molecular nature of the GR therein, and that human tissues harbor multiple isoforms of this protein, some of which (e.g., GRα and GRβ) are the products of alternative splicing of the GR gene (Oakley et al., 1996), while others are generated from a single GR mRNA species by alternative translation initiation (Lu and Cidlowski, 2005). One of the antibodies used in this study (Ab1) was previously shown to recognize some of these isoforms (Lu and Cidlowski, 2005) and is therefore suitable for investigating treatment-specific differences in
molecular properties of the GR in PBMCs. It was found that H$_2$O$_2$ treatment specifically modulated the pattern of immunospecific GR bands, considerably increasing intensity of the 60-kDa species over that of the corresponding band in untreated cells of healthy volunteers. Interestingly, a very similar pattern of immunospecific bands was obtained from PBMCs of pediatric patients suffering from moderate asthma, while mild asthmatics presented a pattern resembling that of healthy subjects (Perišić et al., unpublished data). Since at this moment the precise cellular roles and intracellular localization of different GR isoforms are far from being elucidated, it is premature to speculate on the possible biological significance of this finding. Besides, it should be first established whether the 60-kDa species represents an isoform or a proteolytic product of the GR.

It is demonstrated in this paper that the alterations of GR binding properties and the protein expression pattern induced by H$_2$O$_2$ treatment of PBMCs from healthy donors are very similar to those registered in PBMCs of moderate asthmatic patients in comparison with mild asthmatics and healthy subjects. In contrast, treatment with other oxidants produced different effects or had no influence on GR binding activity and the protein expression pattern. It follows that this study provides preliminary data suggesting that functional alterations of the GR associated with moderate asthma may be mediated by redox mechanisms relying on the oxidative and regulatory actions of H$_2$O$_2$.

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REFERENCES


МОГУЋА УЛОГА ВОДОНИК ПЕРОКСИДА У ФУНКЦИОНАЛНИМ ПРОМЕНАМА ГЛУКОКОРТИКОИДНОГ РЕЦЕПТОРА У АСТМИ

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Познато је да су патогенеза и напредовање хроничне астме повезани са функционалним променама глукокортикоидног рецептора (GR) и дуготрајном инфламацијом дисајних путева, чији су медијатори реакционарне врсте кисеоника и азота. У овом раду тестирали смо хипотезу да функционалне промене GR у астми настају као последица деловања оксиданата. У том циљу спровели смо серию ex vivo третмана мононуклеарних ћелија периферне крви хормоном и смањењем афинитета рецептора према хормону. Промене индукуване водоник пероксидом, укључујући и карактеристичну експресију изоформи GR хормена, биле су врло сличне променама које су раније запажене у периферним лимфоцитима пацијената оболелих од средње тешког облика астме, али не и код благих астматичара и здравих донора. Третман другим примењеним оксидантима произвео је другачије ефekte, и није имао утицаја на GR. Према томе, ова студија је дала прелепинирне податке који указују да су функционалне промене GR повезане са средње тешком астмом вероватно посредоване редокс механизма који се заснивају на оксидативној и регулаторној улози водоник пероксида.