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Alterations in macrophage G proteins are associated with endotoxin tolerance

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Abstract

Previous studies have suggested that endotoxin tolerance induces macrophage desensitization to endotoxin through altered guanine nucleotide regulatory (G) protein function. In the present study the binding characteristics of the nonhydrolyzable GTP analogue GTPγ[^{35}S] to macrophage membranes from endotoxin tolerant and control rats were determined. Membranes were prepared from peritoneal macrophages harvested from rats 72 h after two sequential daily doses of vehicle or *Salmonella enteritidis* endotoxin (100 μg/kg on day 1 and 500 μg/kg on day 2). GTPγ[^{35}S] bound to a single class of sites that were saturable and displaceable in control and endotoxin tolerant macrophage membranes. The maximum specific binding of GTPγ[^{35}S] was significantly ($P < 0.01$) decreased in membranes from tolerant rats compared to control ($B_{max} = 39 \pm 7$ pmol/mg protein in control vs. 11 ± 2 pmol/mg protein in endotoxin tolerant; $n = 5$). There were no significant differences in the K_d values. To determine whether the reduced GTPγS binding was due to decreases in G proteins, macrophage membrane G protein content was determined by western blotting with specific antisera to $G_{i1,2}\alpha$, $G_{i3}\alpha$, $G_s\alpha$, and the β subunit of G. Scanning densitometric analysis demonstrated differential decreases in tolerant macrophage membrane G proteins. $G_{i3}\alpha$ was reduced the most to $48 \pm 8\%$ of controls ($n = 3$), and this reduction was significant compared to those of other G proteins. $G_{i1,2}\alpha$ and Gβ were reduced to $73 \pm 5\%$ ($n = 3$) and $65 \pm 4\%$ ($n = 3$) of control values, respectively. $G_s\alpha$(L) and $G_s\alpha$(H) were reduced to $61 \pm 5\%$ ($n = 3$) and $68 \pm 3\%$ ($n = 3$) of control, respectively. These results demonstrate that endotoxin tolerant macrophages exhibit decreased membrane GTP binding capacity and differential reductions in the content of specific G proteins. The cellular mechanisms leading to such alterations in G proteins and their functional significance in the acquisition of endotoxin tolerance merit further investigation.

Keywords: Guanine nucleotide regulatory protein; Pertussis toxin; Endotoxin; Endotoxin tolerance; GTPγ[^{35}S]

1. Introduction

Activation of macrophages by bacterial endotoxin results in the release of multiple mediators thought to play a major role in the pathogenesis of endotoxic shock. These mediators include a variety of cytokines, reactive oxygen metabolites, and arachidonic acid metabolites [1,2]. Sublethal administration of endotoxin to humans and laboratory animals diminishes macrophage release of certain inflammatory mediators, including arachidonic acid (AA) metabolites [3,4] and produces resistance to lethal shock upon secondary challenge with endotoxin [5–8]. This acquired resistance to endotoxin is referred to as endotoxin tolerance. The molecular mechanisms mediating the refractory response of tolerant macrophages to endotoxin stimulation are not known. Changes in macrophage mediator production in endotoxin tolerance do not appear to be a consequence of changes in endotoxin receptors but may be due to altered intracellular signal transduction events [9–11].

Abbreviations: AA, arachidonic acid; G, guanine nucleotide regulatory proteins; PT, pertussis toxin

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The guanine nucleotide regulatory (G) proteins are a family of heterotrimeric proteins that couple membrane receptors of the serpentine class to certain second messenger generating enzymes and ion channels [12–14]. Pertussis toxin (PT) catalyzes the ADP ribosylation of α subunits of the G_i family of G proteins resulting in its functional uncoupling from membrane receptors [15,16]. The involvement of G proteins in endotoxin activation of macrophages is suggested from observations that PT inhibits endotoxin induced PGE_2 formation in rat mesangial cells [17], TXB_2 and 6-keto-$PGF_{1\alpha}$ production in rat peritoneal macrophages [3], induction of interleukin-1β mRNA in U937 cells [18] and nitric oxide production in murine peritoneal macrophages [19].

Previous studies have demonstrated that endotoxin tolerant macrophages exhibit reduced AA metabolism when stimulated by nonspecific G protein activators such as NaF and GTPγS [3,20]. Additionally, we have demonstrated decreased macrophage membrane GTPase activity from endotoxin tolerant rats [20]. The time of onset of suppressed membrane GTPase activity after a tolerizing dose of endotoxin correlates with the inability of subsequent endotoxin exposure to stimulate in vitro TXB_2 production in macrophages [20]. These observations suggest an association between decreased G protein function and the development of endotoxin tolerance. To determine if the reduced membrane GTPase activity in endotoxin tolerance is a consequence of altered substrate binding affinity or altered number of binding sites, we examined the binding characteristics of GTP to membranes from endotoxin tolerant and control macrophages using the nonhydrolyzable GTP analogue GTPγ[^{35}S]. Since Gi proteins may be involved in endotoxin signal transduction, we determined whether the reduced macrophage membrane GTPase activity in endotoxin tolerance could be due to reduced content of Gi proteins. Immunoblot analysis of macrophage membranes by specific antibodies to $G_{i1,2}\alpha$ and $G_{i3}\alpha$ were performed. Furthermore, to determine whether the content of other G proteins could be altered, immunoblot analysis of $G_s\alpha$, and the β subunit of G were conducted.

2. Materials and methods

2.1. Tolerance induction

All rats used in these studies were viral antibody-free Long Evans male rats (200–250 g) obtained from Charles River (Durham, NC). Rats were maintained in double filters and reverse flow isolators under controlled temperature and illumination and given food and water ad libitum. Tolerance was induced by intraperitoneal injection of *Salmonella enteritidis* endotoxin (DIFCO, Detroit, MI) for 2 consecutive days at doses of 100 and 500 μg/kg body weight, respectively. The experiments were performed 72 h after the final injection of endotoxin.

2.2. Cell culture

Macrophages were harvested by peritoneal lavage from ether-anesthetized rats with RPMI 1640 medium (GIBCO, Grand Island, NY) with L-glutamine containing penicillin (50 U/ml), streptomycin (50 μg/ml), and sodium heparin (10 U/ml). The cells were allowed to adhere for 2 h on 60 mm plates at 37°C in 5% CO_2.

2.3. Preparation of membranes for GTPγS binding

After adhering for 2 h, the cells were washed 3 times with 5% dextrose and lysed by the addition of ice-cold lysing buffer (25 mM Tris-HCl, 1 mM EDTA, pH 7.4). Macrophages were scraped and homogenized in lysing buffer. The suspension was then dounced 13 times and centrifuged at $30\,000 \times g$ at 4°C for 1 h. The pellet was resuspended in lysis buffer, dounced and centrifuged again at $30\,000 \times g$ for 60 min. The pellet was resuspended in membrane buffer (20 mM Tris-HCl, 1 mM EDTA, pH 7.4).

2.4. GTPγ[^{35}S] binding

Equilibrium binding experiments were conducted in incubations containing 20 μl of membrane suspension (2.5 μg protein, 20 mM Tris-HCl, 1 mM EDTA, pH 7.4), 30 μl of buffer containing 20 mM Tris-HCl (pH 7.4), 1 mM EDTA, 1 mM dithiothreitol (DTT), 20 mM $MgCl_2$, 100 mM NaCl, 0.1% Lubrol (Sigma, St. Louis, MO), 0.15 nM GTPγ[^{35}S] (1300 Ci/mmol) NEN, Boston, MA) and varying concentrations of unlabeled GTPγS (2 nM to 2 μM). Equilibrium was reached by 10 min (data not shown). After incubation for 20 min at 30°C, the samples were placed on ice, 1 ml of 20 mM Tris-HCl, 2 mM $MgCl_2$, pH 7.4, and 1 mM GTP was added to each sample, followed by filtration through a 0.45 μ HAWP filter (Millipore, Bedford, MA) which was then washed 3 times with 20 mM Tris-HCl, 2 mM $MgCl_2$, pH 7.4. Nonspecific binding was determined in presence of 100-fold excess unlabeled GTP and was less than 5% of total. Equilibrium binding data were analyzed according to the method of Scatchard [21] using the computer program Ligand [22].

2.5. Preparation of membranes for Western blotting

Adherent peritoneal MØ were washed 3 times with 5% dextrose then lysed and scraped in lysing buffer with protease inhibitors (25 mM Tris-HCl, 1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 0.2 μg/ml pepstatin A, 4°C, pH 7.4). The suspension was incubated on ice for 1 h. It was then dounced 13 times and centrifuged at $1200 \times g$, 4°C for 15 min. The pellet was resuspended in lysis buffer, dounced and centrifuged again at $1200 \times g$. The supernatants from both centrifugations were centrifuged for 60 min at $100\,000 \times g$ at 4°C. The

membrane pellet was resuspended in lysis buffer. Protein determinations were performed using a protein assay kit from BioRad (Richmond, CA).

2.6. Peptide conjugation and immunization

For the generation of specific antisera designated ASC and AII2, synthetic peptides of the sequence RMHLRQYELL, which corresponds to C terminal sequence of $G_s\alpha$, and KNNLKDCGLF, which corresponds to C terminal sequence of $G_{i1}\alpha$ and $G_{i2}\alpha$, were coupled to the carrier protein keyhole limpet hemocyanin (KLH) using gluteraldehyde as described by Goldsmith [23]. To raise antisera designated BCI, the peptide (C)SWDSFLKIWN, which corresponds to the C terminal sequence of β subunits, was coupled to KLH by the cysteine residue using *m*-maleimido-benzoyl-*n*-hydroxy-succinimide ester as described by Green [24]. Polyclonal antibodies were raised in female New Zealand White rabbits according to the procedure described by Green [24]. The ASC polyclonal antiserum specifically recognized both the large and small forms of $G\alpha_s$ and no other G-protein subunits or isoforms in bovine brain. The AII2 antiserum recognized the α subunits of G_{i1} and G_{i2} purified from bovine brain, but not the β or γ subunits, or the α subunits of G_{oA}, G_{oB}, or $G_s\alpha$. The BCI antiserum recognizes only the β subunit of purified G-proteins and only the 27 kDa C-terminal fragment of the β subunit generated after trypsin treatment. Recombinant $G_{i3}\alpha$ and rabbit antisera to $G_{i3}\alpha$ were prepared as previously described [25].

2.7. Gel electrophoresis

One dimensional SDS-polyacrylamide gel electrophoresis was performed according to the method of Laemmli [26]. Membrane samples were electrophoresed through 1.5 mm thick gels with 2.5 cm of a 4.5% acrylamide/bis stacking gel and 16 cm of 12% acrylamide resolving gel. Coomassie blue staining of membrane proteins was routinely performed to ascertain equal protein loading (data not shown).

2.8. Western blot analysis

Proteins were electrophoretically transferred from the SDS gel to a nitrocellulose membrane [27]. Following transfer, the membrane was blocked for 1 h in a solution of 7.5% dried milk in Tris-buffered saline-Tween-20 (TBS-T) (20 mM Tris, 500 mM NaCl, 0.1% Tween-20, pH 7.5). The membrane was subsequently washed with TBS-T (once for 15 min then twice for 5 min each) and incubated overnight with the rabbit anti-G protein antibody. The antibody dilution ranged from 1:1000 to 1:6000 depending on the specific antibody used. The following day, the membrane was washed as indicated above and incubated with a 1:4000 diluted horseradish peroxidase linked donkey anti-rabbit-IgG antibody (Amersham) for 1 h. The membrane was vigorously washed (5 washes, 15 min each) with TBS-T (0.15% Tween-20) then incubated with the ECL detection reagents (Amersham, Arlington Heights, IL). The G protein bands were detected on film by the chemiluminescent reaction of luminol [28]. G proteins co-migrated with partially purified G proteins from the brain [29] and recombinant $G_{i3}\alpha$.

2.9. Densitometric scanning analysis

Western blots were scanned, and the integrated optical densities for the G protein bands were calculated using the software NIH Image. Initially, several experiments ($n = 5–9$) comparing equal amounts of control and tolerant membrane proteins demonstrated reductions in tolerant macrophage membrane G proteins including $G_{q,11}\alpha$. To determine the percent reduction in G protein levels in tolerance, standard curves using three different amounts of control or tolerant membrane proteins were generated. In one experiment, 8, 5.33 and 4 μg of control macrophage membrane proteins were electrophoresed along with 8 μg of tolerant macrophage membrane proteins. The integrated optical densities of the control G protein bands linearly correlated with the amount of protein loaded. The relative amount of tolerant G protein was extrapolated from the linear curve generated using the three control bands. Tolerant G protein levels were expressed as percent of control. Two similar experiment were performed using 8 μg of control proteins with 8, 12 and 16 μg of tolerant proteins.

2.10. Statistical analysis

Statistical significance for the binding data was determined by the Student's *t*-test. Statistical comparison of control and tolerant G protein bands was performed using a paired Student's *t* test with the Bonferroni correction applied to allow multiple comparisons. Statistical comparison among the percent levels of tolerant G proteins was performed using an analysis of variance followed by Tukey's Honestly Significant Difference test. $P < 0.05$ was considered significant. Data are expressed as means $\pm$ S.E.

3. Results

We assessed the quantity of macrophage membrane GTP binding proteins via GTPγ[^{35}S] binding. GTPγ[^{35}S] binding was saturable (Fig. 1A) and displaceable in control and endotoxin tolerant macrophage membranes. Scatchard analysis of the equilibrium binding data demonstrated that GTPγ[^{35}S] binding in nontolerant and endotoxin tolerant macrophage membranes was consistent with a single class of binding sites (Fig. 1B). The data were best fit by a

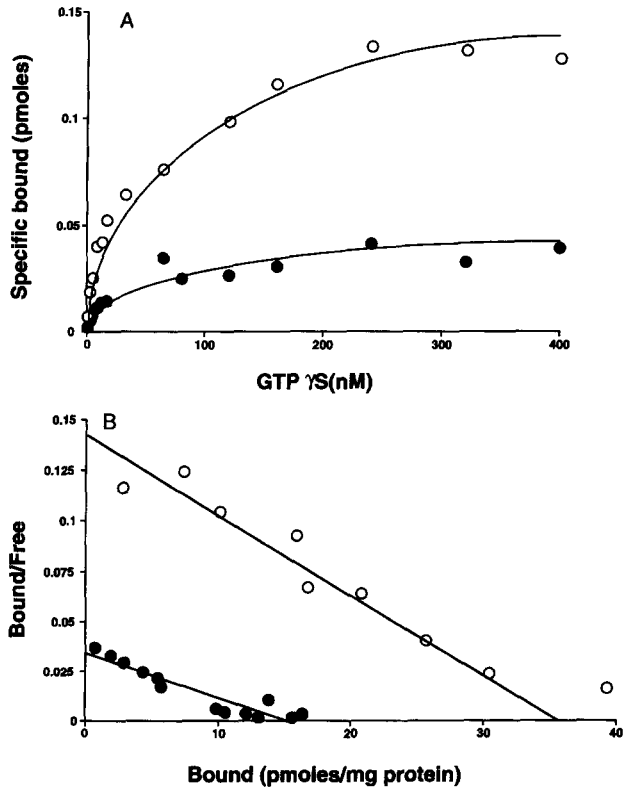

Fig. 1. (A) Representative equilibrium binding data for GTPγS to control (open circles) and tolerant (closed circles) resident peritoneal macrophage membranes. Membranes were incubated with various concentrations of cold and radiolabeled GTPγS. Representative of five experiments. (B) Scatchard analysis of equilibrium binding data in A. The B_{max} values for the control and tolerant groups were 38.5 and 14.5 pmol/mg protein, respectively. The K_d values were 13 and 20 nM, respectively. Representative of five experiments.

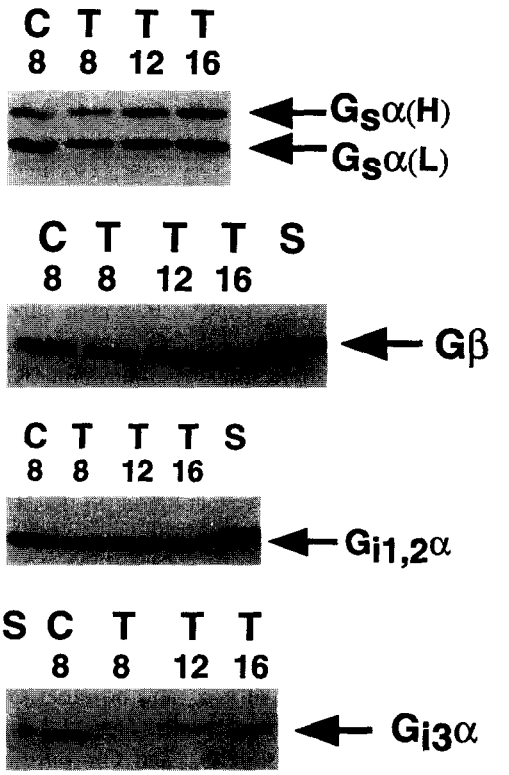

Fig. 2. Representative Western blots of tolerant (T) and control (C) membranes using specific antisera to $G_s\alpha$, $G\beta$, $G_{i1,2}\alpha$ and $G_{i3}\alpha$. 8 μg of control proteins were electrophoresed along with 8, 12, and 16 μg of tolerant proteins. Partially purified G proteins from the brain were used as standards (S) except for the $G_{i3}\alpha$ blot where (S) is recombinant $G_{i3}\alpha$. Representative of three experiments.

single site model. Macrophage membranes from endotoxin tolerant rats exhibited no significant change in binding affinity ($K_d = 20 \pm 4$ nontolerant vs. 18 ± 4 nM endotoxin tolerant; $n = 5$), but a significant decrease ($P < 0.01$) in specific binding capacity ($B_{max} = 39 \pm 7$ nontolerant vs. 11 ± 2 pmol/mg protein endotoxin tolerant; $n = 5$).

Since GTPγ[^{35}S] binding was decreased, we determined if the content of G proteins was decreased. Immunoblotting for $G_{i1,2}\alpha$, $G_{i3}\alpha$, $G_s\alpha$, and the β subunit of G were performed and the blots analyzed by densitometric scanning. Both control and tolerant membranes demonstrated immunoreactivity to all of the $G\alpha$ and β subunits (Fig. 2). The membrane content of all G proteins measured were reduced in tolerant membranes compared to control (Figs. 2 and 3). Densitometric scanning demonstrated that the tolerant membrane $G_{i3}\alpha$ was reduced the most (Fig. 3) to $48 \pm 8\%$ ($n = 3$, $P < 0.05$ vs. control, $P < 0.05$ vs. other G proteins). $G_{i1,2}\alpha$ and $G\beta$ were reduced to $73 \pm 5\%$ ($n = 3$) and $65 \pm 4\%$ ($n = 3$) of control values, respectively. $G_s\alpha$(L) and $G_s\alpha$(H) which represent the low and high molecular weight forms of $G_s\alpha$ were reduced to $61 \pm 5\%$ ($n = 3$) and $68 \pm 3\%$ ($n = 3$) of control, respectively.

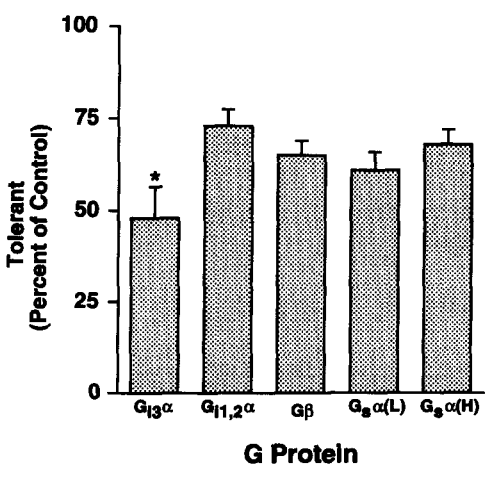

Fig. 3. Tolerant G protein levels expressed as percent of control. The blots were analyzed with a scanning densitometer and the relative amount of control or tolerant G proteins was extrapolated from a curve as described in Section 2. All tolerant G proteins were significantly reduced ($P < 0.05$) compared to control. $G_{i3}\alpha$ was significantly reduced compared to the other G proteins. Data are expressed as mean ± S.E. of three experiments.

4. Discussion

This study demonstrates that endotoxin tolerance is associated with decreased GTPγS binding and G protein content. Endotoxin activation of specific macrophage mediators, such as AA metabolites, appears to be linked to a pertussis toxin sensitive G protein(s) [3,17–20]. Thus, alterations in macrophage G protein function could affect the decrease in these mediators observed in endotoxin tolerance [2,3]. The reduced specific GTPγS binding and the reduced apparent amounts of $G_i\alpha$, $G_s\alpha$, and the β subunit(s) in membranes from endotoxin tolerant macrophages observed in this study are consistent with this notion. These findings also extend our previous observations of decreases in macrophage membrane GTPase activity from endotoxin tolerant rats [20], and reduced AA metabolism in these macrophages in response to the non-specific G protein activators NaF and GTPγS [3,20].

Down-regulation of G proteins has been suggested as a mechanism for heterologous desensitization to certain agonists after prolonged exposure. Reduced G protein function has been implicated in the desensitization of rat aorta after prolonged (1 week) norepinephrine infusions. This desensitization was characterized by reductions in GTPγS binding, contractile responses to NaF, and in the ability of alpha adrenergic receptors to activate $G_i\alpha$ and $G_s\alpha$ proteins [30]. In rat adipocytes, exposure to either the adenosine agonist N^6-phenyl isopropyl adenosine (PIA) or PGE_1 leads to down-regulation of each of the three subtypes of $G_i\alpha$ and decreased inhibition of adenyl cyclase [31]. Since adenosine receptors were not altered by PGE_1 exposure, it was postulated that down-regulation of $G_i\alpha$ proteins were responsible for the resistance of adipocytes to PIA [31]. G_i proteins could mediate many of the cellular effects of endotoxin, including effects on phospholipase A_2, phospholipase C and adenyl cyclase [32]. In the present studies, tolerant macrophage membrane G_i proteins are reduced. This is consistent with the observations that in tolerance, pertussis toxin sensitive pathways, like AA metabolism are suppressed, while other pathways which are not mediated by G_i proteins, like nitric oxide and IL-6 production, are sustained or even increased [33–35]. It is possible therefore, that in endotoxin tolerance, reduced G protein function and content may be responsible for the differential regulation of mediator release.

Heterologous desensitization of post-receptor coupling mechanisms as opposed to endotoxin receptor down-regulation could more readily describe the well established phenomenon of cross tolerance that can be induced between endotoxin and certain non-endotoxin stimuli. This includes cross tolerance to gram-positive bacteria [36], TXA_2 mimetics [37], catecholamine-induced shock [38], drum trauma [39], oxygen toxicity [40], and reperfusion injury in myocardial ischemia [41]. It is unlikely that downregulation of endotoxin receptors is responsible for this broad desensitization. Indeed, several studies have shown that reduced mediator production in tolerance does not correlate with LPS receptor changes [9–11].

The mechanisms leading to the reduced GTPγS binding and G protein content remain to be determined. These reductions are not due to non-specific effects of LPS. The non-toxic and less potent LPS from *Rhodobacter spheroides* [42,43] caused only slight reductions in G protein content (data not shown). In addition, macrophages from rats rendered LPS tolerant by injection of TNF, which is structurally unrelated to LPS, exhibit reduced membrane $G_{i3}\alpha$ content [44]. The reductions in Gi, Gs, Gq (data not shown) and $G\beta$ raise the possibility of a common mechanism for the reduced G protein content, and that other G proteins may also be reduced. The greater reduction in $G_{i3}\alpha$ may be due to an increased susceptibility of $G_{i3}\alpha$ or to the presence of a separate mechanism. This may involve altered gene transcription, mRNA translation or protein degradation.

The present findings are only correlative. They do not show that reduced G protein content and function are the mechanisms of LPS tolerance. Other mechanisms for LPS tolerance, such as the induction of protein repressors [45] or the failure to activate transcription factors [46], have been proposed. Nonetheless, in view of the involvement of G protein pathways in endotoxin activation of macrophages, mechanisms leading to altered cellular G protein function and content and the effects of those changes on macrophage signal transduction merit further investigation.

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