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The influence of stress and methionine-enkephalin on macrophage functions in two inbred rat strains

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Abstract

The aim of our current study was to investigate the effect of acute exposure to electric tail shock stress (ES) and to a stress witnessing procedure (SW), as models for physical and psychological stress paradigms, respectively, on phagocytosis and H_2O_2 production in peritoneal macrophages isolated from Albino Oxford (AO) and Dark Agouti (DA) rats. In addition, we studied the in vitro effects of methionine-enkephalin (ME) on phagocytosis and H_2O_2 production in peritoneal macrophages isolated from both AO and DA rats that had been exposed to ES and SW procedures. The results showed that peritoneal macrophages isolated from DA rats were less sensitive to the suppressive effects of ES and SW than macrophages isolated from AO rats. In vitro treatment of macrophages isolated from AO rats with ME mimicked to some extent the suppressive effects of ES and SW on phagocytosis and H_2O_2 production and additionally diminished H_2O_2 release in macrophages isolated from AO rats previously exposed to ES or SW. ME did not have any effect on phagocytosis in macrophages isolated from DA rats, but changed H_2O_2 production in a concentration-dependent manner. In macrophages isolated from DA rats previously exposed to stress the effect of ME was dependent on the macrophage function tested and the particular stress paradigm employed. Our results emphasise the fact that both beneficial and detrimental effects of stress on immune system functions could be attributed to the individual variations in the macrophage's response to stress mediators.

Keywords: Albino Oxford rats; Dark Agouti rats; Electric tail shock stress; Stress witnessing procedure; Methionine-enkephalin; Peritoneal macrophages; Phagocytosis; Hydrogen peroxide production

Introduction

It is well established that neuronal, endocrine and immune systems communicate with each other via mutual hormones, neuropeptides and cytokines in order to preserve a balanced state of homeostasis (Besedovsky and Del Rey, 1996; Blalock, 1989; Carr and Blalock, 1991; Janković, 1991; Webster et al., 2002). Any disturbance of the homeostatic balance induces a non-specific response termed the stress response (Selye, 1970) that comprises patterned endocrine, autonomic and behavioral changes (Selye, 1936). However, recent reasoning in the field of stress research goes against the notion of the existence of pre-determined patterns of physiological changes but instead points to individual variations in ability to cope with a stressful situation as the key factor that determines the outcome of a stress reaction (Dantzer, 1997; Vogel, 1986). When extrapolated to human and animal physiology, the impact of stress varies greatly depending on genetic background, gender and age (Faraday, 2002; Kudielka et al., 2004; Woolfolk and Holtzman, 1995). Accordingly, genetic background, sex or age differences in neuroendocrine function may translate into differential immunological changes following exposure to specific stressors (Kusnecov and Rabin, 1994).

We have previously shown that five consecutive daily exposures to electric tail shocks profoundly suppressed the humoral immune response in rats, whereas witnessing electric tail shocks actually potentiated the humoral immune response (Stanojević et al., 2003). These opposing changes of the immune response were partially abrogated by systemic

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administration of naloxone, suggesting the involvement of opioid peptides in both stress paradigms. Stress-induced release of opioid peptides protects organisms from painful sensations in dangerous situations. Since pain perception can disrupt effective responses to emergency conditions, activation of intrinsic pain-suppressive mechanisms would be more adaptive for the organism's successful fine-tuning and survival strategies (Terman et al., 1984). However, opioid peptides possess immunomodulatory properties that probably contribute to stress-induced immunological changes (Shavit et al., 1984).

It has been speculated that alterations in the immune response after stress could be ascribed specifically to stress-induced changes in the functions of macrophages, as these cells are involved in the development of humoral and cellular immune responses (Fleshner et al., 1995; Kizaki et al., 1996). We previously reported that the stress-related opioid peptide methionine-enkephalin (ME) non-uniformly regulated hydrogen peroxide (H_2O_2) production in macrophages isolated from two inbred non-MHC compatible rat strains, namely Albino Oxford (AO) and Dark Agouti (DA) rats (Radulović et al., 1995). The aim of our current study was to determine if AO and DA rat strains also differed in their response to stress procedures that are accompanied by the release of opioid peptides. Therefore, we examined if acute exposure to electric tail shock stress (ES) and a stress witnessing procedure (SW), as models of physical and psychological stress paradigms, respectively, affected phagocytosis and H_2O_2 production in peritoneal macrophages isolated from both rat strains. Several lines of evidence suggest that a stressful experience changes the response to a subsequent exposure to stress via a mechanism involving multiple physiological systems (O'Connor et al., 2003). We decided to explore how a previous stress experience could alter the response of immune cells to artificial stressors at the cellular level using opioids in vitro. We accomplished this by studying the in vitro effects of ME on phagocytosis and H_2O_2 production in peritoneal macrophages isolated from AO and DA rats that had been exposed to ES or SW procedure.

Materials and methods

Animals

Young adult male AO and DA rats (60±3 days of age) were obtained from our breeding colony located within the Immunology Research Center "Branislav Janković" in Belgrade. Animals were housed individually in perspex-walled cages with free access to food and water. All procedures involving animals were approved by our Institutional Animal Care and Use Committee and followed guidelines described in the European Community's Council Directive dated 24th November 1986 (86/609/EEC).

Drugs

The opioid pentapeptide methionine-enkephalin (ME, Tyr-Gly-Gly-Phe-Met) and nitro blue tetrazolium chloride (NBT, 2,2′-di-*p*-nitrophenyl-5,5′-diphenyl-3,3′-[3,3′-dimethoxy-4,4′-diphenylene]ditetrazolium chloride) were obtained from Serva (Heidelberg, Germany). Corticosterone (CORT), phenol red, phorbol myristate acetate (PMA), horseradish peroxidase (EC 1.11.1.7.) and zymosan A (from *Saccharomyces cerevisiae*), were purchased from Sigma (St. Louis, MO, USA). Thioglycolate medium and phenol red-free minimal essential medium (MEM) were acquired from the "Torlak" Institute in Belgrade.

Stress procedures

Rats were intraperitoneally injected with 15 ml of thioglycolate medium in order to induce recruitment of circulatory monocytes into the peritoneum and proliferation of resident peritoneal macrophages (Eichner and Smeaton, 1983; Melnicoff et al., 1989). Stress procedures were performed 6 days after injection of the thioglycolate medium. Eight rats from each strain were used for the experiments.

ES was delivered in a soundproof room to which the animals had been moved prior to the stress procedure. During the shock procedure the rats were kept in rectangular perspex-walled boxes (18×9.5×20 cm) containing a semicircular aperture on the lower part of the rear wall that allowed attachment of the electrodes to the rat's tail and prevented movement of its rear paws. Electric shocks were delivered via two silver electrodes separated by 3–4 cm connected to the tail with the aid of electrode paste and adhesive tape. Four rats were simultaneously shocked with the same intensity (55 V, 1 mA). A shock session consisted of eighty 5 s unsignalled shocks (7 electric impulses of 0.3 s duration and 0.6 s pauses) with an average inter-shock interval of 60 s (range 5–120 s). In this way the total duration was 80–90 min. After the shock session the rats were returned back to their colony. As the experimental model of ES involved the exposure of rats to a series of unpredictable, inescapable and uncontrollable electric tail shocks accompanied by early and late opioid-mediated analgesia, it was regarded as both a physical and a psychological challenge that resulted in immunological changes (Maier et al., 1983; Shavit et al., 1984; Sutton et al., 1994). It has been shown that such a procedure induces a several-fold increase in the level of CORT (Ottenweller et al., 1989).

SW involved rats witnessing the procedure of shock delivery to the rats within the ES group. Rats were moved to a soundproof room prior to the stress session at the same time as the rats belonging to the ES group. SW rats remained there during the ES procedure. Specifically, SW rats were kept in their regular cages and witnessed the whole ES procedure. After the ES session, SW rats were returned to their colony. The model of witnessing the ES procedure involved exposure of caged rats to pheromones (Fanselow, 1985) and ultrasonic vocalisation (Knutson et al., 2002) emitted from rats experiencing the ES procedure. Therefore, this experimental design ensures primarily psychological stress for the witnessing rats. Rats witnessing rats experiencing the ES procedure accumulate an elevated level of CORT (Ottenweller et al., 1989) and experience opioid-mediated analgesia (Moynihan et al., 2000). This stress paradigm also produces immunological changes, thereby confirming that physically stressed rats can influence non-

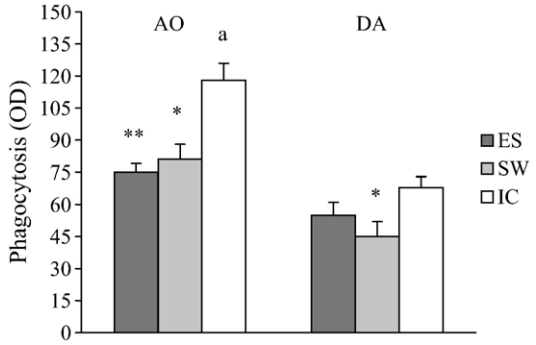

Fig. 1. The effect of exposure to electric tail shock stress (ES) and stress-witnessing procedure (SW) on phagocytosis of zymosan particles in peritoneal macrophages isolated from AO and DA rats. Values represent mean ($n=8$)±S.E.M. Statistically significant differences: (A) *, $p<0.05$ and **, $p<0.01$ vs. the corresponding IC group; [a], $p<0.01$ vs. DA rats.

physically stressed rats via mechanisms involving vocalisation and body odours (pheromones) (Fernandes, 2000; Moynihan et al., 2000).

Serving as controls were intact control (IC) rats which were left undisturbed in their regular cages in the colony.

Isolation of macrophages and their cell surface phenotyping

Seven days after injection of thioglycolate medium [when the majority of the cells in peritoneal exudates are macrophages (Segura et al., 1996)], cells were obtained by peritoneal lavage with 10 ml of MEM. Peritoneal lavages were centrifuged (1200 rpm, 10 min) and the resulting cell pellets were washed three times using phosphate buffer. This procedure allowed adequate recovery of cells (greater than 95% viable, as determined by trypan blue exclusion).

The cells (1×10^7/ml) were stained with mouse anti-rat CD11b IgG-FITC (clone number ED8, Serotec, Oxford, UK) and mouse anti-rat CD68-biotin (clone number ED1, Serotec, Oxford, UK) followed by streptavidin RPE (STAR4A/B) as a second step reagent (Becton Dickinson, San Jose CA, USA). Analysis on a FACScan flow cytometer (Cell Quest software, Becton Dickinson) by means of FSC and SSC of a total of 10^4 flow cytometric events confirmed that the majority (75.8±3.6%) of cells in the peritoneal lavages were ED1+CD11b+macrophages. Cell suspensions were then adjusted to 2.5×10^6 cells/ml (8 individual samples per experimental group, in duplicate) and plated (100 µl/well) in 96-well flat-bottomed tissue culture plates (Linbro, ICN Biomedicals, Aurora, OH, USA). The plates were incubated for 2 h at 37 °C in 95% air - 5% CO_2. Non-adherent cells were removed by washing the plates twice with warmed (37 °C) MEM.

Phagocytosis assay

Phagocytosis was determined according to a modified method of Pick and co-workers (1981). Briefly, macrophages were stimulated with zymosan (125 µg/ml) in the absence or presence of ME (10^{-12}–10^{-6} M) or CORT (10^{-8}–10^{-5} M), in the presence of NBT (0.5 mg/ml) for 30 min at 37 °C in 95% air - 5% CO_2. The cells were then fixed with methanol, the plates were air-dried and optical densities (ODs) were determined at 545 nm. The amount of engulfed zymosan particles was proportional to the reduction of yellow NBT to blue formazan. The results are expressed as OD.

To interpret interactions between the effects of stress (ES or SW) and the effects of ME, numerical data were transformed into percentages (%) in relation to OD values from strain-matched zymosan-stimulated cells from IC rats that represented 100%, according to the following formula:

$$\text{Phagocytosis}(\%) = (\text{OD}_{\text{ME}}/\text{OD}_{\text{zymosan}}) \times 100\%$$

where OD_{ME} represents ODs of the samples from the ES, SW and IC experimental groups stimulated with zymosan and concomitantly treated with ME and $\text{OD}_{\text{zymosan}}$ represents ODs of the samples from IC rats stimulated only with zymosan.

H_2O_2 release assay

H_2O_2 release was determined as previously described (Pick and Mizel, 1981). Briefly, macrophages were primed for

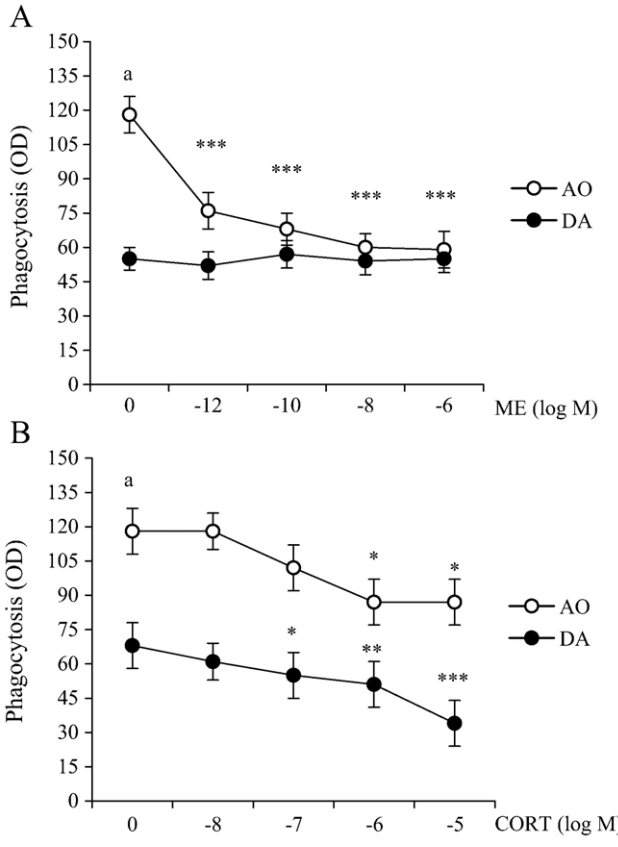

Fig. 2. The effect of in vitro treatment with: (A) 10^{-12}–10^{-6} M of methionine-enkephalin (ME) and (B) 10^{-8}–10^{-5} M of corticosterone (CORT) on phagocytosis of zymosan particles in peritoneal macrophages isolated from AO and DA rats. Values represent mean ($n=8$)±S.E.M. Statistically significant differences: *, $p<0.01$, **, $p<0.001$ and ***, $p<0.0001$ vs. 0; [a], $p<0.01$ vs. DA rats.

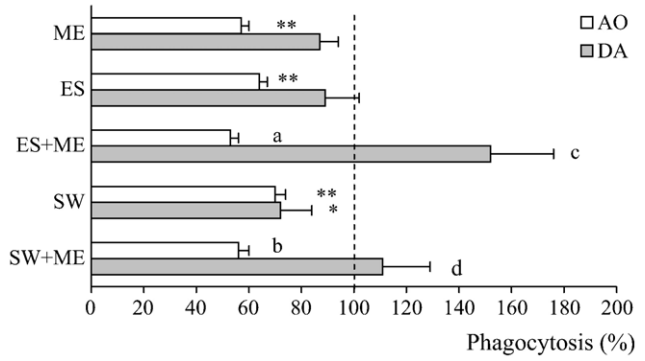

Fig. 3. The effect of in vitro treatment with 10^{-10} M of methionine-enkephalin (ME), exposure to electric tail shock stress (ES) or exposure to stress-witnessing procedure (SW) on phagocytosis of zymosan particles in peritoneal macrophages isolated from AO and DA rats, and the effect of in vitro treatment with 10^{-10} M ME on phagocytosis of zymosan in peritoneal macrophages isolated from AO and DA rats previously exposed to ES (ES+ME) or SW (SW+ME). The values represent % of phagocytosis (mean±S.E.M.) relative to phagocytosis in strain-matched zymosan-stimulated cells from IC rats designated 100%. Statistically significant differences: *, $p<0.05$ and **, $p<0.0001$ vs. 100%; [a], $p<0.0001$ vs. 100% and $p<0.01$ vs. ES; [b], $p<0.0001$ vs. 100% and $p<0.001$ vs. SW; [c], $p<0.05$ vs. 100% and $p<0.01$ vs. ME and $p<0.01$ vs. ES; [d], $p<0.01$ vs. SW.

peroxide production with 100 µl of 25 nM PMA in phenol red solution (10 mM potassium phosphate buffer pH 7, 140 mM NaCl, 5.5 mM dextrose, 0.56 mM phenol red and 19 U/ml of horseradish peroxidase) in the absence or presence of ME (10^{-14}–10^{-6} M) or CORT (10^{-8}–10^{-5} M). PMA was prepared from a stock solution (10^{-2} M in DMSO, stored at −70 °C). The 96-well plates were incubated for 1 h at 37 °C in 95% air - 5% CO_2. Incubations were terminated with 10 µl of 0.5 M NaOH and ODs were determined at 620 nm. The concentration of H_2O_2 in the samples was calculated using standard concentrations of H_2O_2 (1–40 µM). The results were expressed as nM of H_2O_2 produced per mg of cell protein.

To interpret interactions between the effects of stress (ES or SW) and the effects of ME, numerical data were transformed into percentages (%) in relation to OD values from strain-matched PMA-primed cells from IC rats that represented 100%, according to the following formula:

$$H_2O_2 \text{ production } (\%) = (OD_{ME}/OD_{PMA}) \times 100\%,$$

where OD_{ME} represents ODs of the samples from the ES, SW and IC experimental groups primed with PMA and concomitantly treated with ME and OD_{PMA} represents ODs of the samples from IC rats primed only with PMA.

Statistical analysis

Statistical analysis of the data was performed using the StatView II computer programme. The results are presented as mean±S.E.M. The main effects of ES and SW were analysed separately for each strain by one-way ANOVA. The effects of ME in both strains were analysed by one-factor ANOVA for repeated measures (factor: concentration). Fisher's PLSD test was used for post hoc analysis for the evaluation of differences between groups (indicated in the figures with asterisks). Student's un-paired two-tailed *t*-test was used for the evaluation of strain differences

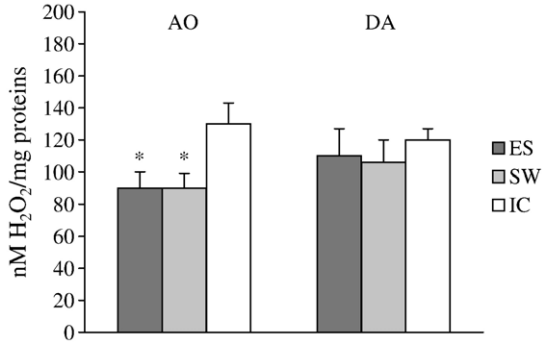

Fig. 4. The effect of exposure to electric tail shock stress (ES) and stress-witnessing procedure (SW) on phorbol myristate acetate (PMA)-primed H_2O_2 production in peritoneal macrophages isolated from AO and DA rats. The values represent mean ($n=8$)±S.E.M. Statistically significant differences: *, $p<0.05$ vs. the corresponding IC group.

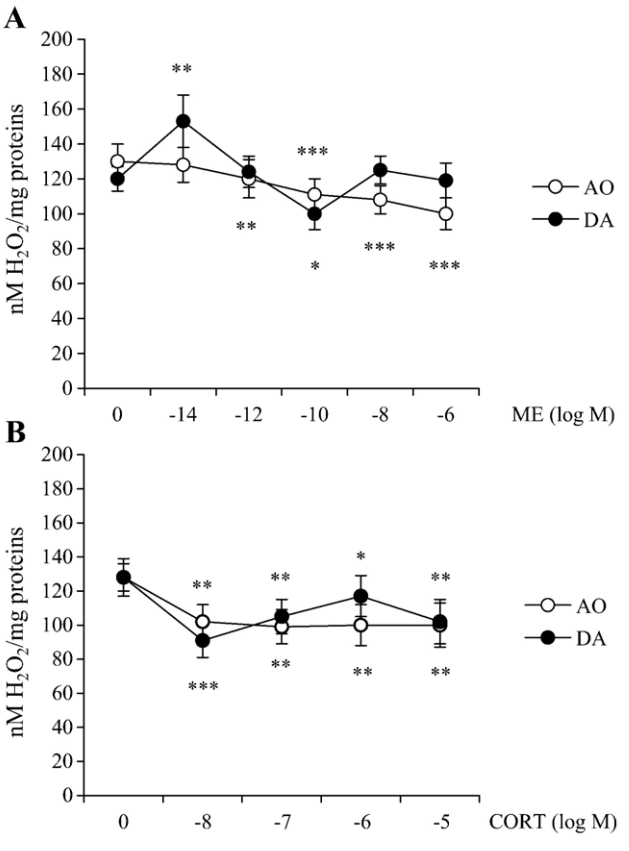

Fig. 5. The effect of in vitro treatment with: (A) 10^{-14}–10^{-6} M of methionine-enkephalin (ME) and (B) 10^{-8}–10^{-5} M of corticosterone (CORT) on phorbol myristate acetate (PMA)-primed H_2O_2 production in peritoneal macrophages isolated from AO and DA rats. The values represent mean ($n=8$)±S.E.M. Statistically significant differences: *, $p<0.05$, **, $p<0.01$ and ***, $p<0.0001$ vs. 0.

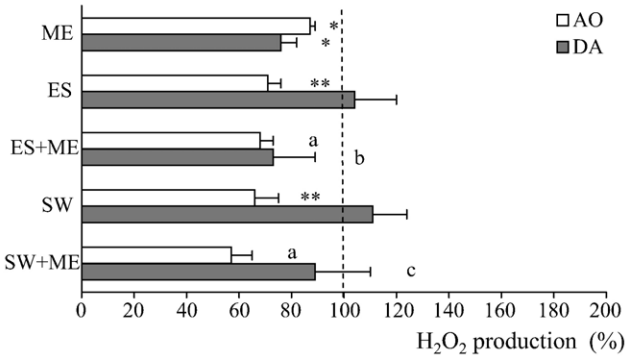

Fig. 6. The effect of in vitro treatment with 10^{-10} M of methionine-enkephalin (ME), exposure to electric tail shock stress (ES) or stress-witnessing procedure (SW) on phorbol myristate acetate (PMA)-primed H_2O_2 production in peritoneal macrophages isolated from AO and DA rats, and the effect of in vitro treatment with 10^{-10} M ME on PMA-primed-H_2O_2 production in peritoneal macrophages isolated from AO and DA rats previously exposed to ES (ES+ME) or SW (SW+ME). The values represent % of H_2O_2 production (mean±S.E.M.) relative to H_2O_2 production in strain-matched PMA-primed cells from IC rats designated 100%. Statistically significant differences: *, $p<0.05$ and **, $p<0.001$ vs. 100%; [a], $p<0.0001$ vs. 100% and $p<0.0001$ vs. ME; [b], $p<0.05$ vs. 100% and $p<0.05$ vs. ES; [c], $p<0.05$ vs. ME and $p<0.05$ vs. SW.

between IC groups in each test. Differences were regarded as statistically significant if the p value was <0.05.

Results

The effect of stress and/or treatment with ME on macrophage phagocytosis

Acute exposure to ES diminished phagocytosis of zymosan in macrophages isolated from AO rats whereas acute exposure to SW diminished phagocytosis in macrophages isolated from both rat strains (Fig. 1). All phagocytosis data were compared to phagocytosis in macrophages isolated from strain-matched IC rats. Macrophages isolated from IC rats belonging to the AO strain phagocytosed more zymosan than macrophages isolated from IC rats belonging to the DA strain. ME (at all concentrations) decreased phagocytosis in macrophages obtained from AO rats, but had no influence on macrophages isolated from IC rats belonging to the DA strain when compared with strain-matched samples only stimulated with zymosan (Fig. 2A). In contrast, CORT diminished phagocytosis in macrophages isolated from both strains (Fig. 2B). In further analysis, numerical data were expressed as percentages of phagocytosis relative to phagocytosis in strain-matched zymosan-stimulated macrophages isolated from IC rats (designated 100%).

Post hoc analysis of the data (expressed as relative values) confirmed results obtained with non-transformed data. It revealed that exposure to ES or SW and treatment with 10^{-10} M ME independently decreased phagocytosis in macrophages isolated from AO rats (Fig. 3). However, a combined exposure to ES or SW with 10^{-10} M ME resulted in a reduction of phagocytosis comparable to that induced solely by treatment with 10^{-10} M ME in macrophages isolated from AO rats. In macrophages isolated from DA rats phagocytosis was not influenced by ES or by treatment with ME, but a combined exposure to ES and treatment with ME significantly increased phagocytosis when compared with ES or ME alone (Fig. 3). Exposure to SW decreased phagocytosis in macrophages isolated from DA rats. However, SW in combination with ME significantly increased phagocytosis, suggesting that ME attenuated the suppressive effect of SW.

The effect of stress and/or treatment with ME on macrophage H_2O_2 production

Both ES and SW reduced H_2O_2 production only in macrophages isolated from AO rats (Fig. 4), when compared to H_2O_2 production in macrophages isolated from strain-matched IC rats. Macrophages isolated from IC rats of both strains produced similar amounts of H_2O_2 as judged by the phenol red assay. ME (10^{-12}–10^{-6} M) reduced H_2O_2 release from macrophages isolated from IC rats belonging to the AO strain. In contrast, both an increase (at 10^{-14} M ME) and a decrease (at 10^{-10} M ME) in H_2O_2 production was observed in macrophages isolated from IC rats belonging to the DA strain (Fig. 5A) when compared to strain-matched samples primed only with PMA. CORT effectively decreased H_2O_2 release from macrophages isolated from both rat strains (Fig. 5B). In further analysis, numerical data were expressed as percentages of H_2O_2 production relative to H_2O_2 production in strain-matched PMA-primed macrophages from IC rats (designated 100%). Treatment with 10^{-10} M ME or exposure to ES or SW significantly decreased H_2O_2 production in macrophages isolated from AO rats (Fig. 6) [the same as obtained by analysis of the raw data (Figs. 4 and 5A)]. Moreover, exposure to ES or SW followed by treatment of macrophages isolated from AO rats with 10^{-10} M

Table 1
Schematic illustration of the effects of exposure to electric tail shock stress (ES), stress witnessing processing (SW), in vitro treatment with methioninenkephalin (ME), or combinations of prior exposure of rats to ES or SW with in vitro treatment with 10^{-10} M of ME(ES+ME, SW+ME), on phagocytosis and hydrogen peroxide (H_2O_2) production in peritoneal macrophages isolated from Albino Oxford (AO) and Dark Agouti (DA) rats

	Phagocytosis		H_2O_2 production	
	AO	DA	AO	DA
ES	↓	∅	↓	∅
SW	↓	↓	↓	∅
ME	↓	∅	↓	↑↓
ES+ME	↓ (Effect of ME)	↑ (Interaction)	↓ (Additive)	↓ (Effect of ME)
SW+ME	↓ (Effect of ME)	∅ (Abolition)	↓ (Additive)	∅ (Abolition)

ME further decreased H_2O_2 release when compared to 10^{-10} M ME (Fig. 6). This implied additive effects of stress and ME. In DA rats, analysis of the relative changes in H_2O_2 production in macrophages confirmed that treatment with 10^{-10} M ME decreased H_2O_2 production, whereas exposure to ES or SW had no effect. In macrophages isolated from DA rats exposed to ES and subsequently treated with 10^{-10} M ME, the decrease in H_2O_2 production was most likely due to the effect of 10^{-10} M ME. However, the suppressive effect of 10^{-10} M ME on H_2O_2 production was abolished in macrophages isolated from DA rats that had previously been exposed to SW (Fig. 6). Table 1 summarises all the results.

Discussion

Our study demonstrated different sensitivity to both physical and psychological stress paradigms (represented by ES and SW, respectively) and different responses to ME in two inbred rat strains in two macrophage function tests. The macrophage functions that were tested mimicked the sequential activation steps upon introduction of a pathogen or an inflammatory stimulus in the body. Peritoneal macrophages isolated from DA rats were less sensitive to the stress events compared to peritoneal macrophages from AO rats. The significance of the animal strain used to study the effect of stress on macrophage function has been previously documented (Churin et al., 2003) and is based upon variations in neurochemical, behavioral and metabolic characteristics among different strains of laboratory animals (Brodkin et al., 1998; Dimitrijević et al., 2001).

Strain-dependent sensitivity to stress is probably, but not exclusively, connected with strain-dependent differences in the activity of the hypothalamic–pituitary–adrenal (HPA) axis (Chrousos, 2000). DA rats express minimal or no significant circadian variations in plasma CORT levels, suggesting impaired HPA axis regulation in this strain (Brodkin et al., 1999). The reason for the latter is most likely due to the reduced volume of zona reticularis in adrenal tissue that may contribute to a reduced capacity for glucocorticoid synthesis or storage (Wilkinson et al., 1999). However, the basal CORT level in DA rat plasma is approximately 300 ng/ml (our unpublished observation). Furthermore, both DA and AO rats exhibit a robust HPA axis response upon stress (Stefferl et al., 1999; Živković et al., 2005) and dexamethasone equally suppresses paw inflammation in both rat strains (Stanojević et al., 2002). Taken together, all these observations imply that both basal and stress-induced differences in the immune system between these rat strains are not entirely linked to the differences in the HPA axis. In agreement with the above, Moynihan et al., 2000 showed that stress-induced immunological alterations occur in the absence of a detectable change in the levels of glucocorticoids. More to the point, CORT at physiological and/or elevated (stressed) levels decreased phagocytosis and H_2O_2 production in macrophages from both rat strains in our study although ES and SW generally influenced macrophage functions in AO rats.

Immune cells from DA rats produce a greater quantity of pro-inflammatory cytokines such as interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α), interleukin (IL)-1, IL-6 and IL-17 than cells from AO rats (Arsov et al., 1995; Mensah-Brown et al., 2005; Miljković et al., 2006; Stošić-Grujičić et al., 1995; Vukmanović et al., 1990). Considering the pivotal role of cytokines in the regulation of macrophage functions, a different cytokine balance in these rat strains probably contributes to different macrophage stress responses. Higher cytokine levels in DA rats could compensate the stress-induced decrease in phagocytic ability and subsequent H_2O_2 production in peritoneal macrophages.

Macrophages from AO and DA rats respond differently to ME (Radulović et al., 1995), a factor that may contribute to their different sensitivity to stress. In our study ME suppressed phagocytosis only in macrophages isolated from AO rats that initially phagocytosed significantly more zymosan than macrophages from DA rats. Our observation is in line with the findings that the effects of ME vary greatly depending on basal cellular activity. For example, ME is capable of suppressing superoxide anion production only in human neutrophils with high basal superoxide anion release (Marotti et al., 1990). Accordingly, ME was demonstrated to increase and decrease natural killer cell activity in individuals with low and high basal activities, respectively (Oleson and Johnson, 1988). Conversely, PMA-stimulated macrophages isolated from both AO and DA rats produced comparable amounts of H_2O_2 (our present study), suggesting that the mechanism of ME-induced changes in H_2O_2 production was not dependent on the degree of macrophage activation, but rather on inherited differences in macrophage biology between the two rat strains. Despite the fact that genome linkage explorations have identified several loci and their interactions responsible for the variability in stress responses of several recombinant inbred strains of rats (Dumas et al., 2000), the genetic loci controlling strain differences in the responsiveness to biological effects of morphine and opioid peptides have not been mapped, nor have the phenotypic bases for these differences been discovered (Eisenstein et al., 1995). However, dose-dependent effects of ME (low-dose potentiation and high-dose suppression of immune functions) as revealed by H_2O_2 production in macrophages isolated from DA rats are observed after in vivo treatment regimes (Janković and Marić, 1987) and in vitro tests (Foris et al., 1986, 1987; Pasnik et al., 1999). Although ME is considered to be a ligand for endogenous δ opioid receptors (Radulović et al., 1995), one of the possible explanations for its dose-dependent effects is that ME binds to δ opioid receptors with high affinity and binds to μ opioid receptors with low affinity (Fischer, 1988), implying that higher concentrations of ME lead to loss of receptor selectivity and a switch to suppressive cellular signalling pathways. In particular, a high dose of ME potentiated weak paw edema in AO rats whereas a low dose of ME suppressed severe paw edema in DA rats. Both observations were ascribed to genotype-dependent differences in the expression of specific types of opioid receptors (Stanojević et al., 2002). Thus, ME activated μ and κ opioid receptors in cells within subcutaneous tissue of AO rats and δ opioid receptors in DA rats.

Interestingly, ME suppressed both phagocytosis and H_2O_2 production in macrophages isolated from AO rats, but differently affected phagocytosis and H_2O_2 production in

macrophages isolated from DA rats. Phagocytosis and H_2O_2 release are indicators of the phagocytotic and secretory capabilities of macrophages, respectively, but both parameters actually gauge oxidative products after the respiratory burst (Pick and Mizel, 1981; Pick et al., 1981). The main difference emanates from the divergence in the route of cell activation, since zymosan particles bind to receptors for the iC3b component of complement on macrophages, initiate phagocytosis and consequently initiate oxidative burst, whereas PMA directly activates protein kinase C and stimulates oxidative burst without phagocytosis (Chanok et al., 1994; Fallman et al., 1993; Johnston and Kitagawa, 1985). Our results suggested that ME suppressed macrophage functions in AO rats irrespective of the manner by which the cells were stimulated. In contrast, in macrophages isolated from DA rats the cell activation mechanism appears important for the action of ME.

Previous observations have shown that systemic application of ME abrogated some of the immunological changes induced by stress, suggesting interference by ME with endogenous stress mediators at several possible levels (Marotti et al., 1996). The reasoning in the current study was that in vivo exposure to neuroendocrine mediators released during stress, including opioid peptides, creates a hormonal milieu that may change macrophage reactivity to subsequent ME challenge in vitro. The results obtained herein employing macrophages isolated from AO rats revealed that only ME at a high concentration (10^{-10} M) interfered with the suppressive influence of ES and SW mediators in the case of H_2O_2 production, therefore further decreasing the suppression level irrespective of the stress paradigm employed. Considering that in AO rats ME mimicked the effects of ES and SW on both macrophage functions and additively suppressed H_2O_2 production, one could speculate that endogenous ME may be implicated in the effects of ES and SW in these rats. Alternatively, ME could mimic the effects of neuroendocrine mediators (other than ME) and act synergistically, further suppressing H_2O_2 production. In macrophages isolated from DA rats, a combination of ES and ME potentiated phagocytosis despite the fact that ES and ME did not have any individual effect. This suggests that ES mediators "primed" macrophages in vivo allowing changes in their phagocytic ability after addition of ME in vitro. In contrast, the addition of ME counteracted the suppressive influence of SW on macrophage functions making the macrophages resistant to the suppressive effects of 10^{-10} M ME. Our results strongly suggest that in less stress-responsive DA rats a clear distinction could be made between the effects of physical and psychological stress paradigms by the addition of the stress-related opioid peptide ME to macrophages in vitro. In conclusion, exposure of DA rats to ES and SW produces subtle but different changes in macrophage functions that are not only detectable but are also regulated by the presence of ME.

We have previously shown that the basal level of endogenous ME modulates the oxidative burst of macrophages and that the mechanism varies depending on the rat strain (Radulović et al., 1995; Vujić-Redić et al., 2000). Of relevance to our current study are the findings of Tsukada et al. (2001) who showed that footshock stress induced a significantly greater increase in the levels of endogenous opioid peptides (up to 800 fM in plasma) than a milder form of psychological stress (up to 300 fM). In addition, it has been shown that stress involving mediators corticotropin-releasing hormone and catecholamines (Cabot et al., 2001; Mousa et al., 2004) enhances the release of biologically active pro-enkephalin-derived peptides from macrophages which can then act in an autocrine/paracrine manner (Saravia et al., 1998).

In vitro supplementation of ME to cells that have already been exposed to different stress mediators, including ME, may result in the activation of specific δ opioid receptors (Radulović et al., 1995), the activation of more than one type of opioid receptor (Vujić et al., 2004) or in the activation of opioid receptor complexes in cell membranes (Boyadjieva et al., 2004). It appears that opioid receptors can form complex hetero-oligomers with other receptors, such as β-adrenergic receptors (Jordan et al., 2001). Therefore, ME could interact with mediators released during stress at the level of their membrane receptors or by interfering with their intracellular signalling cascades.

It can be generally concluded that genotype-dependent differences in the secretion of cytokines and (probably) neuroendocrine hormones contribute to the different sensitivity of macrophages isolated from AO and DA rats to mediators of stress, including ME. Our results emphasise the fact that both beneficial and detrimental effects of stress on immune system functions could be attributed to the individual variations in the macrophage's response to stress mediators.

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