Published in final edited form as:

Toxicol Appl Pharmacol. 2015 December 1; 289(2): 313–322. doi:10.1016/j.taap.2015.09.013.

A physical/psychological and biological stress combine to enhance endoplasmic reticulum stress

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Abstract

The generation of an immune response against infectious and other foreign agents is substantially modified by allostatic load, which is increased with chemical, physical and/or psychological stressors. The physical/psychological stress from cold-restraint (CR) inhibits host defense against *Listeria monocytogenes* (LM), due to early effects of the catecholamine norepinephrine (NE) from sympathetic nerves on β 1-adrenoceptors (β 1AR) of immune cells. Although CR activates innate immunity within 2 h, host defenses against bacterial growth is suppressed 2–3 days after infection (Cao and Lawrence 2002). CR enhances inducible nitric oxide synthase (iNOS) expression and NO production. The early innate activation leads to cellular reduction-oxidation (redox) changes of immune cells. Lymphocytes from CR-treated mice express fewer surface thiols. Splenic and hepatic immune cells also have fewer proteins with free thiols after CR and/or LM, and macrophages have less glutathione after the *in vivo* CR exposure or exposure to NE *in vitro*. The early induction of CR-induced oxidative stress elevates endoplasmic reticulum (ER) stress, which could interfere with keeping phagocytized LM within the phagosome or re-encapsulating LM by autophagy once they escape from the phagosome. ER stress-related proteins, such as glucose-regulated protein 78 (GRP78), have elevated expression with CR and LM. The results indicate that CR enhances the unfolded protein response (UPR), which interferes with host defenses against LM. Thus, it is postulated that increased stress, as exists with living conditions at low socioeconomic conditions, can lower host defenses against pathogens because of oxidative and ER stress processes.

Keywords

Oxidative stress; Beta Adrenoceptor; Neuroimmunology; Stress response; *Listeria*; autophagy

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Conflict of interest statement

No authors have any conflicts of interest.

Introduction

Stress responses have evolved to increase an organism's chance of survival, by strengthening certain defense mechanisms required for "fight or flight" responses, but not without a price. It is known that an organism's susceptibility to infection is increased by stress; the aim of our study was to elucidate the cellular mechanisms involved in this sympathetic nervous system regulation of immunity to *Listeria monocytogenes* (LM). Cold (4°C) restraint (CR) has been a model to elicit a physical and psychological stress response (Paré and Glavin, 1986; Glavin et al., 1994). Physical and psychological stresses are associated with lower socioeconomic status and poorer health (Alder and Rehkopf, 2008). Previously, it was reported that CR for 1 h induces factors, mainly from 6-hydroxydopamine-sensitive nerves of BALB/c mice, and that these neurotransmitters diminish immunologic defenses against the intracellular bacterium LM (Cao et al., 2002). CR interferes with the bacteriostatic/bactericidal response 2–3 days after infection, which was observed as an increased LM burden in the liver and spleen of BALB/c mice (Cao and Lawrence, 2002). Previous reports indicate that CR induces release of catecholamine, which signals via β 1-adrenoceptors (β 1AR) to impair host defenses by altering innate immune mechanisms on immune cells from BALB/c or FVB/N mice (Cao et al., 2003a; Emeny et al., 2007). The current *in vivo*, *ex vivo* and *in vitro* study utilized the mouse model to investigate the mechanisms associated with the CR-induced effects.

Within the first 48–72 h of an LM infection, innate immune-defenses orchestrate the host-pathogen response and optimally prime Th1 adaptive immune cells to begin regulating the elimination of the bacteria. CR administered just before LM infection delays this response, prolonging and increasing the bacterial burden (Cao and Lawrence, 2002; Emeny et al., 2007). Peak levels of bacteria usually occur around day 3 under normal experimental conditions; the CR-stressed mice also exhibit peak LM burden on day 3 in the liver and spleen, but between days 2–3, there is less control of bacterial propagation (Cao and Lawrence, 2002). This early delay in bacterial killing indicates that CR interferes with the cellular and molecular mechanisms that normally control the intracellular propagation of bacteria and/or which facilitate the switch from innate to adaptive processes. We, therefore, performed a series of experiments to investigate oxidative and intracellular changes that might be implicated in stress-induced immunologic suppression immediately following CR treatment and then subsequently for 48 h after LM infection.

Materials and Methods

Mice, cold-restraint (CR) treatment, and *Listeria monocytogenes* infection

All experiments were performed using mice housed at the AAALAC-approved Wadsworth Center animal facility, in accordance with Institutional Animal Care and Use Committee guidelines. All mice were maintained on a 12 h light/dark cycle with lights on from 7 AM to 7 PM. Although all experimental mice were 6–12 weeks of age, experimental comparisons always used mice within 2–3 weeks of age, which have no significant immune differences due to age. BALB/c mice were acquired from Taconic (Germantown, NY). β 1AR^{-/-} and β 2AR^{-/-} mice, bred on the FVB/NJ (FVB) background, were kindly provided by Dr. B. Kobilka (Stanford Univ). Wildtype (WT)-FVB mice were obtained from Jackson Laboratory

(Bar Harbor, ME). For CR treatment, control mice were left in their original cages undisturbed, while mice subjected to CR were individually restrained in well-ventilated plastic 60-ml syringes at 4°C for 1 h in the dark. CR represents a physical and a psychological stress. CR was performed between 8 and 11 AM on day 0; the mice were infected with LM immediately after CR treatment. Mice were intravenously injected with a sub-lethal dose of LM ($2-3 \times 10^3$ colony-forming units (CFU)/i.v. injection for FVB and $3.5-5 \times 10^3$ CFU/i.v. for BALB/c mice). The experiments were approved by the Wadsworth Center's internal review board.

Serum preparation and measurement of nitric oxide

Peripheral blood was collected into 1.7-ml Eppendorf tubes from BALB/c mice. After clot formation (overnight at 4°C), serum was collected following centrifugation. Nitrite levels were measured with sera from BALB/c mice ($n=3$ /group/time point) without CR and at 2, 6 and 24 h after CR using the Griess assay (Green LC et al). Griess reagent (100µl) (a 1:1 mixture of 1% sulfanilamide (*p*-aminobenzenesulfonamide) in 5% H_3PO_4 and 0.1 % N-(1-naphthyl)ethylenediamine dihydrochloride in H_2O) (Sigma-Aldrich, St. Louis, MO) was added (v/v) to diluted serum and standard ($NaNO_2$, Sigma) in 96-well plates. Plates were incubated at room temperature for 10 min, and absorbance was measured at 550 nm in a plate reader and concentration was determined from the standard curve.

Detection of surface levels of protein sulfhydryls on peripheral blood lymphocytes of WT-FVB, $\beta 1AR^{-/-}$ and $\beta 2AR^{-/-}$ FVB mice

Following CO_2 anesthesia, whole blood was obtained by cardiac puncture from control mice and mice subjected to CR stress ($n = 4-6$) and placed into heparinized tubes. Blood (50 µl) was washed twice with 0.1% NaN_3 in PBS, stained for analysis of surface sulfhydryls (R-SH) with 20 µM AlexaFluor488 maleimide (Invitrogen, Carlsbad, CA) in 200 µl PBS for 15 min, on ice, in the dark, and washed twice with PBS. Peripheral lymphocytes were then stained with surface antibodies (CD45, CD3, CD19) in the presence of Fc Block for 20 min on ice. Pharmlyse (Pharmingen) was used to lyse the red blood cells after which cells were analyzed by flow cytometry. The acquisition and analysis of all specimens were performed on a BD FACSCalibur using CellQuest software at the Wadsworth Center Flow Cytometry Core.

Respiratory Burst Assay

Splenic and hepatic macrophages from three mice per strain (WT-FVB and $\beta 1AR^{-/-}$ FVB) on day 2 after LM infection $\pm$ CR were used to measure respiratory burst of mitochondrial activity using the dihydrorhodamine 123 (DHR-123, Molecule Probes) assay, which quantifies the conversion of non-fluorogenic DHR-123 to fluorescent rhodamine-123. Briefly, cells were resuspended in 0.5 ml sample buffer (SB; Hanks Balanced Salt Solution [HBSS] without Ca^{2+} or Mg^{2+} [Sigma]) containing 0.1% BSA and 1mM EDTA and 18 µl of a solution of DHR (29 mM in DMSO) diluted 1:10 with SB. Following a 15 min incubation in a 37°C water bath, cells were centrifuged at $800 \times g$ and resuspended in 1ml PharmLyse (BD BioSciences) and incubated for 10 min at room temperature. After centrifugation and one wash with SB, cells were resuspended in 0.5 ml SB and analyzed by flow cytometry.

Analysis of protein thiols with biotin-maleimide labeling and Western blots

Cells were processed for biotin-maleimide labeling as previously described (Bayer et al, 1987). In brief, spleens and livers were removed from BALB/c mice 48 h after LM infection $\pm$ CR and then were homogenized on ice with Tris-HCl buffer (50 mM, pH 7.6) containing 150 mM NaCl, 2 mM EDTA, 1 mM Na-orthovanadate, 5 mM NaF, 1% NP-40, 10 μ g/ml aprotinin, 10 μ g/ml leupeptin, and 1 mM PMSF. Lysed homogenates were centrifuged for 15 min at 15,000 $\times$ g at 4°C. Biotin-maleimide (50 μ M) was added to each preparation immediately and kept 1 h on ice with occasional shaking. The same concentration of protein (15–25 μ g) from each mouse was diluted with one-half volume of sample buffer (SB) containing 9% (w/v) sodium dodecyl sulfate (SDS), 30% (v/v) glycerol, 2% (v/v) 2ME, and 2.25% (w/v) bromophenol blue in 62.5mM Tris-HCl buffer (pH 6.8). SDS polyacrylamide gel electrophoresis (PAGE) was performed in 10% separating gels, after which the gels were placed upon nitrocellulose paper and subjected to blot transfer as described (Towbin et al., 1979). The blotted proteins were then blocked with 5% fish gelatin in PBST (0.05% Tween-20) overnight at 4°C and incubated with streptavidin-horseradish peroxidase (HRP) conjugate (1:20,000 in blocking buffer) for 1h at 4°C with shaking. The blot was developed by incubating with super signal/chemiluminescent substrate (Pierce Biotechnology, Inc., Rockford, IL) for 5 min and was assayed with a LAS-1000plus (Fuji Film, USA).

Intracellular GSH content of macrophages after *in vivo* CR treatment or *in vitro* norepinephrine (NE) exposure

Peritoneal exudates were obtained from control or CR-treated mice and cultured on glass coverslips with or without 1 μ M NE in the presence or absence of LPS (100 ng/ml) for 48 h. GSH was detected using a mouse monoclonal IgG1 antibody (8.1GSH) against the GSH adduct with *N*-ethylmaleimide (NEM), as previously described (Ault & Lawrence, 2003). Briefly, cells were fixed in cold 4% paraformaldehyde in PBS (20 min), treated with cold 10 mM NEM in PBS (20 min), postfixed with cold 1% glutaraldehyde in PBS (1 h), washed twice with PBS, dehydrated in an ethanol series, and embedded in LR White. Cut sections were treated with the 8.1GSH antibody, which is specific for GS-NEM and no other NEM adducts (Messina et al., 1987), and followed with a 1:100 dilution of 10-nm gold particles conjugated to goat anti-mouse IgG antibodies (Ted Pella, Inc., Redding, CA). Stained sections were viewed at 80kV with a Zeiss 910 transmission electron microscope.

Immunostaining of Western Blots

Equal amounts of extracted protein from each mouse were prepared and assayed by SDS-PAGE as described earlier. The separated proteins were then transferred to Immobilon-P membranes (EMD-Millipore). The membrane blots were first probed with a primary antibody overnight at 4°C. The primary antibodies used included: anti-BiP/GRP78 (BD Transduction Laboratories (BDTL), 610979; 1:250); anti-mouse iNOS (BDTL, 610329; 1:4000); anti-calreticulin (BDTL, 612137; 1:5000); anti-caspase 12 (BD Pharmingen, 551430; 1:2000); anti-CHOP (Cell Signaling Technology, 5554; 1:1000); anti-GRP94 (Cell Signaling Technology, 2104; 1:1000), anti p-JNK (Santa Cruz Biotechnology, SC-6254; 1:200); anti -ATF6 (Imgenex-Biotech, IMG-273; 1:150); and anti-Ero1 α (Santa Cruz Biotechnology, SC-100805; 1:200). Primary antibodies were followed with horseradish

peroxidase (HRP)-conjugated rabbit anti-mouse or goat anti-rabbit antibody. Anti-GAPDH, β -actin and β -tubulin primary antibody (Sigma-Aldrich, St. Louis, MO) was used for loading control. The blots were developed by incubation with Super Signal/chemiluminescent substrate (Pierce Biotechnology, Rockford, IL) for 5 min and were assayed with a LAS-3000plus imager (Fuji). The results are reported as “normalized fold expression”, which was calculated as the chemiluminescence value of the indicated analyte divided by that of the loading control.

RNA Isolation

Total RNA was isolated from control and experimental mouse spleens and livers, stored at -80°C , with the aid of a Qiagen Midi RNeasy kit obtained from Qiagen, Inc. (Valencia CA). Spleens and liver sections were homogenized with RLT reagent containing 2-ME using a rotor-stator homogenizer. The homogenate was cleared of debris by a centrifugation at $4,000 \times g$ for 20 min at room temperature. Each supernatant was applied to a midi column and spun into the column by centrifugation ($4,000 \times g$) at room temperature. Columns were then washed with RW1 buffer and then with 2 washes of RPE buffer according to the Qiagen protocol manual. RNA was eluted from the column with two aliquots of 250 μl of RNase free water. The RNA was further purified on a Qiagen mini column, which included an “On-Column DNase digestion” for removal of contaminating genomic DNA. After several washes the RNA was eluted from the mini column with 60 μl of RNase free water and an aliquot was taken to measure the absorbance at 260 and 280 nm using a Beckman DU640 spectrophotometer. RNA was considered pure if the 260/280 ratio was >1.8 . To check for RNA degradation, 0.5–1 μg of each sample was run by electrophoresis on a 1% agarose gel. Separated RNA was visualized by staining with ethidium bromide and results were documented using a UV transilluminator and Polaroid camera. Roughly a 2/1 ratio of 28S to 18S ribosomal RNA indicated intact, non-degraded RNA.

RNA Quantitation by Real-Time RT-PCR

TaqMan gene expression kits from Applied Biosystems Inc. (Foster City, CA) were employed to provide relative quantitation of gene expression levels. Real-time TaqMan RT-PCR was carried out according to the manufacturer’s protocol. Amplifications were performed according to kit specifications using an Applied Biosystems 7500 Real-Time PCR instrument. The TaqMan gene expression kits used were the following: IL-10, Mm00439616_m1; IL-17A, Mm00439619_m1; TGF β 1, Mm00441724_m1, TRAIL, Mm00437174_m1; IFN α 12, Mm00616656_s1; IFN β 1, Mm00439546_s1; IFN α 4, Mm00833969_s1; IL-13, Mm00434204_m1; Bcl2, Mm00477631_m1; HMGB1, Mm00849805_g1; GDI-2, Mm01599124_g1; Mcl, Mm00501738_m1; Cox2, Mm00478374_m1; CEACAM1, Mm00442360_m1; Mycn, Mm00476449; and GAPDH, Mm99999915_g1. All experimental gene expression results were normalized to the sample’s own internal expression of GAPDH. Gene expression was calculated according to the equation: Gene Expression = $2^{-(\text{Exp Ct} - \text{GAPDH Ct})} \times 1000$.

Two-dimensional electrophoresis (2DE)

Isoelectric focusing (IEF) was performed on each ReadyStrip IPG strip (17 cm, pH4–7, linear, Bio-rad, Hercules, CA, USA) with IPGphor IEF system (Bio-Rad, USA). Samples

containing 200 µg protein from spleens (LM ± ACR) were made up to 300 µl with rehydrating buffer (8 M urea, 2 M thiourea, 4% CHAPS, 65 mM dithiothreitol (DTT), 0.33% carrier ampholyte (pH 4 to 6 and pH 5 to 7, respectively)). After the active rehydration for 12 h, IEF was conducted at 250 V for 1 h, 500 V for 1 h, 1,000 V for 1.5 h, 5,000 V for 2 h, 8,000 V for 0.5 h, and 8,000 V to reach a total of approximately 70kVh. After IEF, the strips were equilibrated first, in equilibration buffer I (1% DL-DTT, 50 mM Tris-HCl (pH 6.8), 6 M urea, 30% glycerol, and 2% sodium dodecyl sulfate (SDS) for 15 min and second, in equilibration buffer II (2.5% iodoacetamide instead of 1% dithiothreitol in equilibration buffer I) for another 15 min. Finally, the second dimension was performed using 12% acrylamide gels, and the 2D-PAGE gels were stained with SyproRuby (Bio-rad, Hercules, CA, USA).

MS/MS protein analysis

Protein bands of interest were isolated from SyproRuby stained SDS-PAGE gels, destained, and partially dehydrated with 0.1 M Tris (pH 8)–50% (v/v) acetonitrile for 30 min at 37°C, and this was followed by 5 min in a sonicating water bath to ensure removal of free stain from the gel pieces. Gel pieces were then dried in a Speed-Vac at ambient temperature under vacuum for approximately 30 min and rehydrated in 0.1M Tris (pH 8)–0.05% (w/v) *n*-octylglucoside (Sigma) containing modified trypsin (0.1 µg/µl: Boehringer Mannheim) or endoproteinaseGlu-C (protease V8) (Roche Molecular Biochemicals). Proteins were digested for 18–24 h at 37°C. After digestion, samples were mixed with an equal volume of 0.1 M Tris (pH 8)–50% (v/v) acetonitrile, and dithiothreitol was added to a final concentration of 1 mM before incubating at 50°C for 20 min. Free sulfhydryl groups of the digested peptide fragments were alkylated by adding iodoacetamide (Sigma) to a final concentration of 3–4 mM and incubating at 37°C for 40 min. Peptide fragments were recovered by extracting the gel pieces twice with 60% (v/v) acetonitrile–0.1% (v/v) trifluoroacetic acid and shaking for 40 min at room temperature. Peptide extracts were combined and concentrated 10-fold in a Speed-Vac vacuum centrifuge prior to mass spectrometry (MS) analysis. Ion-trap mass spectrometry was performed with a Finnigan LCQ DECA (Thermo Finnigan, San Jose, CA) equipped with a new objective Pico View nano-ESI source (New Objective, Inc., Cambridge, MA). Peptide digests were loaded on a Michrom peptide CapTrap (MichromBioResources, Inc., Auburn, CA) plumbed to a 75-µm ID New Objective Pico Frit liquid chromatography(LC) column packed with 5 cm Aquisil C₁₈. Peptide digests were desalted on the CapTrap and then eluted from the reverse-phase column with a gradient of 2–70% (v/v) acetonitrile–0.1% (v/v) formic acid vs 0.1% (v/v) formic acid in water over 70 min. The LC gradient was generated by an ABI 140B dual-syringe pump (ABI/Kratos, Foster City, CA) and split to a flow of less than 500 nl/min with a home-built flow splitter. Data-dependent LC–mass, spectroscopy (MS), zoom scans, and LC–MS–MS of eluting peptides were acquired in repetitive 3-s cycles by the triple-play software of the LCQ system. MS/MS spectra data sets were used to query the mouse genome (NCBI accession no. NC–000962) with the SEQUEST protein identification software.

Statistical Analysis

Data from two or more independent experiments were combined and differences between two experimental groups (where $n \geq 3$) were determined using the one-way ANOVA and Tukey's post hoc test or *t*-test SigmaStat (Jandel Scientific, San Rafael, CA). Effects of CR stress or $\beta 1$ AR deficiency on host immunity were considered significant when *P*-value was < 0.05 .

Results

CR induces oxidative stress

Immediately after CR, blood was isolated and assayed with flow cytometry. The B ($CD19^+CD3^-$), T ($CD3^+CD19^-$), and non-B/T ($CD3^-CD19^-$) lymphocytes ($CD45^{hi}SSC^{low}$) were separately assayed for surface thiols with AlexaFluor488-maleimide. The acute (1 h) CR exposure decreased the number of free sulfhydryls on the surface of peripheral blood lymphocytes from wild-type FVB/N mice (WT-FVB), but not FVB mice lacking $\beta 1$ -AR or $\beta 2$ -AR (Fig. 1), which partially supports the suggestion that CR interference with host defenses against BALB/c mice may be due to oxidative stress. CR suppressed host defenses against LM with WT-FVB mice but not $\beta 1$ -AR $^{-/-}$ mice (Emeny et al. 2007). Interestingly, there also was no significant loss of lymphocyte thiols with cells from $\beta 2$ -AR $^{-/-}$ mice but this strain was more susceptible to CR suppression suggesting additional mechanisms are involved.

GSH levels of peritoneal macrophages after the *in vivo* CR treatment or *in vitro* exposure to norepinephrine (NE)

Peritoneal macrophages were isolated from CR-treated or non-stressed BALB/c mice and cultured with and without LPS and/or NE. LPS activation of macrophages is known to lower GSH levels, and LPS was used as a control in these experiments. Glass-adherent cells were assayed after 48 h for expression of GSH by immunoelectron microscopy. Both the *ex vivo* analysis of CR and the *in vitro* exposure to NE (1 μ M) severely decreased the levels of GSH in all intracellular compartments (Fig. 2).

Cellular proteins with free thiols

To assess if CR had a relatively ubiquitous effect on cellular proteins expressing an available thiol, protein lysates from mice treated with CR and/or LM were treated with biotin-maleimide and assayed with Western blot analysis. An example of PAGE analyses of liver and spleen homogenates indicated that LM, CR and LM + CR lowered the availability of free thiols (Fig. 3), suggesting that these stresses induced an oxidizing environment causing loss of protein thiols.

Serum nitric oxide levels and iNOS expression after CR

Nitric oxide (NO) is a multifunctional biomolecule involved in a variety of physiological and pathological processes. NO production by macrophages is produced by iNOS. CR rapidly induced a significant amount of NO at 2 h to 6 h (Fig. 4), which was related to

upregulation of iNOS in the liver (Fig. 5). Splenic expression of iNOS was not significantly affected by CR (data not shown).

Respiratory burst in spleen and liver macrophages at 48 h after LM infection $\pm$ CR

Respiratory burst was not different between the LM and LM + CR groups with splenic or hepatic macrophages (Fig. 6). However, after LM + CR, mitochondrial activity of $\beta 1AR^{-/-}$ macrophages from livers had significantly greater production of ROS than the macrophages from the livers of the WT-FVB mice, which may explain why $\beta 1AR^{-/-}$ mice kill LM more efficiently after CR (Emeny et al., 2007).

Grp78 expression is enhanced early after CR

Endoplasmic reticulum (ER) stress is accompanied by transcriptional activation of the ER chaperone glucose-regulated protein 78 (Grp78/BiP); therefore, Grp78 expression was used as a measure of ER stress (Luo et al., 2003). Similar to the expression of iNOS, Grp78 expression was rapidly upregulated by CR (Fig. 7).

ATF6 expression after LM infection and/or CR treatment

Cleavage of AFT6 from its p90 form to p50 is another sign of ER stress. There was a significant increase in p50 at 48 h after CR + LM (Fig. 8). The liver gets a higher LM burden than the spleen (Cao et al. 2003), which is likely why the liver has greater expression of ER stress. Along with the increased expression of the p50 form of AFT6, there was an increase in other ER stressrelated proteins, GRP78, p-JNK, CHOP, and caspase-12 (Fig. 9). GRP94, a Ca^{2+} binding chaperone associated with ER stress GRP94 (Supplemental Fig.1) and ER oxidoreductin 1 alpha (Ero1 α) (Supplemental Fig. 2), which affects oxidative folding of proteins, also were upregulated. The ER enzyme protein disulfide isomerase (PDI), which aids disulfide shuffling for protein folding, was not significantly affected by CR (data not shown).

CR alters the 2D gel protein profile of spleen cells from LM-infected mice

At 24 h after LM infection $\pm$ CR, 2D gel electrophoresis was employed to analyze protein expression patterns of spleens (Fig. 10). Some of the proteins with increased or decreased presence were assayed by MS/MS. Some proteins upregulated by CR were heat shock protein (84kDa), the calcium binding protein calreticulin, and the nuclear phosphoprotein SET (32kDa). Two proteins down-regulated by CR were identified as enolase 1 or alpha non-neuron/GDP dissociation inhibitor 2 protein and high mobility group box 1 (HMGB1). HMGB1 (Supplemental Fig. 3) and calreticulin (Supplemental Fig. 4) were further assayed with Western blot analysis and confirmed to be down- and up-regulated, respectively.

mRNA levels altered by CR-treatment following 24 and 48 hr of LM infection

Gene expression of Bcl2, Ceacam1, Cox2, GDI-2, Hmgb1, IFN- α 4, IFN- α 12, IFN- β 1, IL-10, IL-17A, Mcl, Mycn, TGF- β , and TRAIL were assessed. Splenic (Table 1) and hepatic (Table 2) preparations from BALB/c mice (LM and CR + LM groups, n=4) were analyzed by RT-PCR at 24 and 48 h after LM infection. Relative expression was calculated as described. At 24 h, TRAIL and IFN α 12 expression in the spleen and TGF- β and Ceacam1 in

the liver were significantly diminished by CR. At 48 h, TGF- β expression in the spleen was significantly suppressed by CR.

Discussion

Herein, the focus was on CR modulation of immune cells since it was previously demonstrated that CR suppresses host defense against LM (Cao et al., 2002), and CR was less suppressive in $\beta 1AR^{-/-}$ mice (Cao et al., 2003a) or in WT-FVB mice when $\beta 1AR^{-/-}$ immune cells were adoptively transferred (Emeny et al., 2007). This suggested that the CR, which signaled $\beta 1AR^{+}$ cells causing the suppression, was affecting immune cells and not endothelial or epithelial cells. CR induced immediate depletion of surface thiols on circulating T and B cells, and it has been known that loss of surface thiols on lymphocytes lessens their ability to be activated for proliferation (Duncan and Lawrence, 1988). However, the early effects of CR could involve innate and/or adaptive immune cells since antigen-specific T cell activation requires antigen-presenting cells (APCs). Studies have shown that the first day of bacterial infection is a critical time when pathogen-specific $CD8^{+}$ T lymphocytes are activated, after which they can proliferate and acquire effector function independent of further antigenic encounter (Mercado 2000). $CD4^{+}$ T cells can enhance $CD8^{+}$ T cell activities, but they are not required for the primary $CD8^{+}$ T cell response to LM (Sun et al., 2004). However, it was previously reported that CR does not inhibit $CD8^{+}$ T cell expansion and activation to LM in WT mice (Emeny et al., 2007). CR also did not prevent increased numbers or amounts of neutrophils, macrophages, NK cells, T cells, IL-6 or IFN γ , which are cells and factors associated with defense against LM (Cao et al., 2003b). TGF- β has been reported to aid protection against LM (Nakane et al., 1996), so the TGF- β decline, especially the early (24 h) decline in the liver (Table 2), may partially be responsible for the CR-induced suppression. The CR-induced decline of TGF- β and IL-10 suggest that increased Treg activity is unlikely to be responsible for the CR-induced suppression. TRAIL expression also was decreased by CR, but TRAIL null mice are resistant to listeriosis (Zheng et al., 2004), and TRAIL is not needed for $CD8^{+}$ T cells to mount defenses against LM (Badovinac et al., 2006; Sacks and Bevan, 2008); furthermore, $CD4^{+}$ T cells from $\beta 1AR^{-/-}$ mice expressed less TRAIL than those from WT mice. The question remains as to what immune mechanisms are modified by this acute physical and psychological stress, which is why early immune cell alterations were evaluated.

There has been no evidence of CR-induced changes in lymphocyte proliferation, based on antigen-specific $CD8^{+}$ T cell expansion or the absolute numbers of the peripheral leukocyte populations. The early loss of cellular thiols, including glutathione, may have modified immune development enough to cause an inhibition that was not apparent until 48 h when the increased LM burden was observed. The early expression of iNOS and elevated level of NO, which is known to affect mitochondrial alterations associated with triggering of ER stress (Xu et al., 2004), is why we examined biochemical changes due to CR treatment and possible alterations of intracellular mechanisms.

The suppression of host defenses against LM could relate to loss of GSH in macrophages. Both CR and NE caused loss of GSH in isolated macrophages. The GSH level of macrophages and other APCs is known to affect differential development of naïve helper T

cells (nTh or Th0 cells). Lower GSH levels of APC preferentially promote Th2 development (Peterson et al., 1998; Murata et al., 2002), and host defenses against LM are known to require Th1 and CD8⁺ T cell mediated immunity. Recovery of GSH and other thiols by 72 h after the CR and LM infection might explain how the killing of LM begins to become equivalent with and without the CR stress of WT mice (Cao et al., 2002).

It seems paradoxical that CR enhances early NO production and yet is posited to interfere with host defense against LM since NO is used as a means to kill intracellular LM. However, as for the GSH levels, the temporal relationships may be inappropriate for optimal killing of LM, in that the elevated NO level is occurring before the immune cells have bound or phagocytosed LM. Early NO and iNOS expression could lead to a premature compensatory anti-inflammatory response, which usually occurs to prevent excessive cell-mediated immunity once the pathogen numbers are lowered or eliminated. Interestingly, CR-treated $\beta 1AR^{-/-}$ hepatic monocytes/macrophages produced more ROS, as determined by respiratory burst at 48 h, than that of WT cells, which are less able to kill LM after CR. When stimulated *in vitro* with TLR2 or TLR4 ligands, M1 macrophages, which promote bactericidal activity, are switched by 24 h to M2 macrophages, which phagocytose but have less killing potential than M1 inflammatory macrophages (Banerjee et al., 2013). A premature shift of M1 to M2 macrophages could be the cause of the declined killing of LM by CR. Interestingly, the level of HMGB1, which promotes inflammation (Keyel, 2014) and TLR2 signaling (Kwak et al., 2015), was lowered by CR.

LM is able to lysis out of the phagosome and propagate in the cytoplasm of macrophages; controlling LM expansion is dependent on autophagy, i.e. re-encapsulating cytosolic LM in a phagolysosome before it is able to utilize the cell's actin to escape the phagocyte and infect more cells (Birmingham et al., 2008). HMGB1, which had suppressed expression after CR, is known to aid autophagy (Deretic, 2011). Cell stress, which was apparent by increased expression of HSPs, calreticulin, GRP78, and cleavage of ATF6 is suggested to relate to ER stress and the unfolded protein response (UPR), which would interfere with autophagic activity needed to intracellularly form phagosomes. The early effects of NO are suggested to make phagocytes more susceptible to intracellular propagation of LM due to elevated proteins associated with UPR, which interferes with proteins needed for synthesis of phagosomes.

Increased physical and psychological stresses are occurrences that are more common with individuals living in lower socioeconomic conditions (Dowd et al., 2009; Zajacova et al., 2009; Kim et al., 2013). Such circumstances may explain higher prevalence of ill health with increased socioeconomic stresses (Braveman et al., 2010; Rook, 2013; Schreier and Chen, 2013), including increased incidence of infections with catecholamine inhibiting Th1 and M1 cell activities and promoting Th2 and M2 cells. Although short-term stress can enhance certain aspects of immunity (Dhabhar, 2014), the acute effects of CR, a physical and psychological stress, was able to interfere with host defenses against an intracellular pathogen, which is known to be more pathogenic due to its ability to escape into the cytosol. Intrinsic to catecholamine binding of cell surface βARs is the immediate activation of G-protein coupling to intracellular effectors such as adenylate cyclase (AC). These enzymes then act to produce the second messenger cAMP (by AC) and subsequent downstream

molecules such as protein kinase A (PKA) and (extracellular signal regulated kinase) ERK2. Enhanced AC activity preferentially promotes type-2 immune activities (Lacour et al., 1994; Klein-Hessling et al., 2003; Tokoyoda et al., 2004), which limits Th1 and M1 functions. PKA is known to inhibit the initiation of autophagy (Stephan et al., 2009). Thus, NE-mediated β 1AR effects via oxidative and ER stress may skew macrophage differentiation and interfere with M1 killing.

In addition to the physical and psychosocial stresses associated with low socioeconomic status, it may be mechanistically relevant to point out that the environment also likely has higher levels of metal toxicants, such as lead. Similar to CR, lead (Pb) preferentially skewed naïve T cells toward Th2 cells, and cAMP was implicated in this skewing (Heo et al., 1998). Additionally, like CR, the interference with host defense against LM by Pb did not appear until day 2–3 after infection (Dyatlov and Lawrence, 2002). Thus, psychosocial stress and metal stressor Pb may share common pathways, including oxidative stress and catecholamine. Pb and psychological stress have been shown to generate additive, synergistic or potentiated effects (Cory-Slechta et al. 2008), and β -AR functions may be involved. β -AR regulation is suggested to be an important component of the link between psychosocial stresses and health (Euteneuer et al., 2012).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

The authors thank the Immunology Core of Wadsworth Center for their assistance with the flow cytometry and the Mass Spectrometry Lab. The immunoelectron microscopy was performed in the Wadsworth Center's EM Core facility. This work was supported, in part, by NIEHS grant U01 ES016014.

All authors were employed by Wadsworth Center or Health Research Inc of New York State Department of Health at the time of data collection.

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Highlights

- Cold-restraint (physical/psychological stress) induces early oxidative stress
- The oxidative stress relates to catecholamine signaling beta-adrenoceptors
- Physical/psychological stress combine infection enhancing inflammation
- Endoplasmic reticulum stress interferes with host defenses and autophagy

Figure 1. CR affects cellular thiols

Flow cytometric analysis of surface thiols (sulfhydryls) on blood lymphocytes immediately after CR with the impermanent fluorescent thiol specific probe (Alexafluor488-maleimide). CD3 and CD19 were used to assess gated lymphocytes (CD45^{hi}/SSC^{low}) after CR treatment of WT, $\beta 1AR^{-/-}$ and $\beta 2AR^{-/-}$ FVB/NJ mice (n=4–6). Results are presented as geometric mean fluorescence intensity (GeoMFI). A significant ($p < 0.05$) loss of surface thiols after stress treatment is noted (*) for T and B cells from WT-FVB mice by one-way ANOVA and Tukey's post hoc test.

Figure 2. Intracellular GSH of peritoneal macrophages is reduced after *in vitro* exposure to NE or prior *in vivo* CR treatment

Peritoneal macrophages were harvested from BALB/c mice immediately after no stress or CR stress. The cells were cultured on glass coverslips with and without *in vitro* LPS (100 ng/ml) and/or NE (1 µM). Shown are immunoelectron micrographs of gold particles (10 nm) in macrophages from CR stressed or non-stressed mice; control was no CR or NE (A). Gold beads, which represent amounts of GSH, were counted in different sections of the preparations to achieve number of beads/µm² (B).

Figure 3. PAGE analysis of cellular proteins with free thiols
Liver and spleen homogenates from untreated control mice and mice LM infected and/or CR stressed were assayed for proteins with thiols able to be bound by biotin-maleimide.

Figure 4. Elevated levels of serum nitric oxide (NO) were quantified after CR

Serum was collected at 2, 6, and 24 h after CR-stressed or from non-stressed BALB/c mice, and the concentration was determined by Griess assay. Significant amounts of NO production were observed at 2h–6h after CR. Asterisk indicates a significant difference (* $p < 0.05$, ** $p < 0.01$) from non-stressed controls.

Figure 5. Elevated levels of iNOS protein expression were observed after CR

The protein expression of iNOS was determined by Western blot analysis ($n = 3$) of total protein lysate from liver and spleen of stressed and non-stressed mice. Tubulin was used as a loading control. Significant amount of iNOS expression support the CR-induced NO induction. Asterisk indicates a significant difference (* $p < 0.05$, ** $p < 0.001$) from non-stressed controls.

Figure 6. Respiratory burst of splenic and liver macrophages at 48 h after LM infection $\pm$ CR
 Spleen and liver single cell suspensions were gated on macrophages by light scatter and assayed by flow cytometry for production of ROS with rhodamine 123. LM (n=4) and LM + CR (n=4) BALB/c mice were assayed. The symbol # indicates a significant difference between WT and $\beta 1AR^{-/-}$ strains, while * indicates a difference observed due to CR stress treatment.

Figure 7. Analysis of GRP78, a molecular marker of ER stress

The protein expression of GRP78 was determined by Western blot analysis ($n = 3$) of total protein lysates from livers and spleens of non-stressed BALB/c mice and CR-stressed BALB/c mice after 2 h, 6 h, and 24 h. β -actin was used as a loading control; inserts show examples of the blots. Asterisks indicate significant differences (* $p < 0.05$, ** $p < 0.001$) from nonstressed controls.

Figure 8. ATF6 expression in the spleens (A) and livers (B) after 48 h LM infection and/or CR treatment

The data is presented as mean $\pm$ SEM. Significant differences from control (non-stressed) values are indicated by * $p < 0.05$ or ** $p < 0.001$.

Figure 9. Expression of ER stress related proteins GRP78 (A), p-JNK (B), CHOP (C), and Caspase-12 (D) in the livers after 48 h LM infection and/or CR treatment
The data is presented as mean $\pm$ SEM. Significant differences from control (non-stressed) values are indicated by * $p < 0.05$ or ** $p < 0.001$.

Figure 10. 2D gel analysis of splenic homogenates 48 h after LM infection $\pm$ CR
Narrow pH range isoelectric focusing 2D gel (pH 4.0–7.0) of soluble proteins (0.2 mg) was loaded onto the gel and further separated by SDS-PAGE. More than 100 protein spots were visible by SyproRuby staining for the homogenates from the LM infected mice (A) and the mice CR-treated prior to infection (B). Five protein spots differences were assayed by MS/MS (1, HSP84; 2, calreticulin; 3 SET; 4, enolase1, and 5 HMGB1).

Table 1LM infection $\pm$ CR effects on RNA transcripts from spleens

Gene	24 h		48 h	
	LM	LM + CR	LM	LM + CR
IL-10	2.22 $\pm$ 0.14	1.91 $\pm$ 0.18	4.61 $\pm$ 0.17	4.31 $\pm$ 0.14
IL-17A	3.73 $\pm$ 0.87	3.57 $\pm$ 0.72	0.2 $\pm$ 0.05	0.08 $\pm$ 0.03
TGF- β	184.04 $\pm$ 3.51	181.3 $\pm$ 8.78	125.2 $\pm$ 1.95	107.4 $\pm$ 1.52*
TRAIL	41.77 $\pm$ 1.01	34.08 $\pm$ 2.56*	18.08 $\pm$ 0.51	17.73 $\pm$ 0.63
IFN α 12	0.03 $\pm$ 0.002	0.02 $\pm$ 0.004*	0.02 $\pm$ 0.003	0.03 $\pm$ 0.001
IFN β 1	1.11 $\pm$ 0.10	0.81 $\pm$ 0.14	0.42 $\pm$ 0.06	0.48 $\pm$ 0.08
IFN α 4	0.16 $\pm$ 0.02	0.10 $\pm$ 0.02	0.49 $\pm$ 0.008	0.05 $\pm$ 0.005
IL-13	0.15 $\pm$ 0.02	0.11 $\pm$ 0.01	0.04 $\pm$ 0.003	0.03 $\pm$ 0.01
Bcl2	46.25 $\pm$ 0.95	44.20 $\pm$ 2.86	22.18 $\pm$ 0.67	20.62 $\pm$ 1.26
HMGB1	39.68 $\pm$ 2.05	41.13 $\pm$ 1.43	22.33 $\pm$ 0.70	21.00 $\pm$ 0.77
GDI-2	127.26 $\pm$ 1.52	132.01 $\pm$ 2.0	99.14 $\pm$ 3.05	98.16 $\pm$ 2.07
Mcl	18.05 $\pm$ 0.98	16.67 $\pm$ 0.95	10.47 $\pm$ 0.26	11.36 $\pm$ 1.52
Ceacam1	4.72 $\pm$ 0.15	5.31 $\pm$ 0.28	3.87 $\pm$ 0.12	3.75 $\pm$ 0.16
Cox2	8.48 $\pm$ 0.94	8.33 $\pm$ 0.66	2.79 $\pm$ 0.16	2.68 $\pm$ 0.42
Mycn	1.13 $\pm$ 0.12	1.07 $\pm$ 0.11	0.75 $\pm$ 0.01	0.77 $\pm$ 0.07

* Values represent relative expression calculated with the formula $2^{-(\text{Exp.Ct} - \text{GAPDH Ct})} \times 1000 \pm \text{S.E.M}$

The asterisk (*) indicates significance according to Student's t-test, $p < 0.05$.

The numbers represent the average of 4 experiments, $n = 4$.

Table 2LM infection $\pm$ CR effects on RNA transcripts from livers

Gene	24 h		48 h	
	LM	LM + CR	LM	LM + CR
IL-10	0.03 $\pm$ 0.0037	0.02 $\pm$ 0.0045	0.26 $\pm$ 0.013	0.26 $\pm$ 0.032
IL-17A	0.013 $\pm$ 0.0019	0.014 $\pm$ 0.002	0.01 $\pm$ 0.0041	0.009 $\pm$ 0.0064
TGF- β	8.52 $\pm$ 0.73	5.98 $\pm$ 0.17*	11.57 $\pm$ 0.92	12.32 $\pm$ 0.51
TRAIL	19.85 $\pm$ 2.01	16.31 $\pm$ 0.53	14.80 $\pm$ 1.55	16.03 $\pm$ 0.78
Ceacam1	16.42 $\pm$ 0.66	12.55 $\pm$ 0.51*	ND	ND

Values represent relative expression calculated with the formula $2^{-(\text{Exp.Ct} - \text{GAPDH Ct})} \times 1000 \pm \text{S.E.M}$

The asterisk (*) indicates significance according to Student's t-test, $p < 0.05$.

The numbers represent the average of 4 experiments, $n = 4$.