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Effects of acute cold exposure on plasma inflammatory and lipid biomarkers related to cardiovascular disease risk

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Abstract

Purpose: Acute cold exposure (ACE) stimulates metabolism but data evaluating inflammatory and lipid biomarkers related to cardiovascular disease in response to ACE are lacking. Therefore, we investigated the relationship between ACE and inflammatory and lipid biomarkers.

Methods: Twenty subjects underwent 30 min of ACE with blood drawn: 1) pre-ACE (baseline), 2) at 30 min ACE, and 3) 2 h post-ACE. Plasma was analyzed for 10 cytokines (monocyte chemoattractant protein-1 (CCL2), interleukin (IL)-1β, IL-2, IL-4, IL-6, IL-8, IL-10, granulocyte-macrophage colony stimulating factor (GM-CSF), tumor necrosis factor-alpha (TNF-α), interferon-gamma (INFγ)), 9 acute phase proteins (ferritin, fibrinogen, procalcitonin, serum amyloid A, serum amyloid P, tissue plasminogen, C-reactive protein, haptoglobin, α-2-macroglobin), and 5 lipid biomarkers (triglycerides, nonesterified fatty acids (NEFA), low- and high-density lipoprotein (LDL, HDL), and total cholesterol). Heart rate, whole body oxygen consumption (VO_2), energy expenditure (EE), shivering sensations, and pain were measured prior and during ACE.

Results: ACE increased heart rate by 11%, shivering sensations by 4-fold, pain by 2-fold, VO2 by 50%, and EE by 52%. IL-1β increased after 30 min ACE by 24% (p=0.048) and 2 h post ACE by 65% (p=0.01). Alpha-2-macroglobin increased after 30 min ACE by 16% (p=0.005) and returned to baseline levels 2 h post-ACE. HDL increased at 2 h post-ACE by 15% (p=0.034). Sex differences were noted between IL-2, IL-6, IL-8, ferritin, α-2-macroglobin and HDL.

Conclusions: These findings indicate ACE increases EE and modifies inflammation, the acute phase response, and lipid metabolism. Long-term risk of CVD needs further exploration to assess the risks or benefits of ACE. Future studies with diverse subjects varying in age and body composition and studies aimed to determine the effectiveness of ACE as compared to diet and exercise are warranted.

Abbreviations: ACE : Acute cold exposure, ACTH: Adrenocorticotropic hormone, ANOVA: Analysis of variance, BMI: Body mass index, BW: Body weight, CCL2: Chemokine (C-C motif) ligand 2 or known as MCP-1 (monocyte chemoattractant protein-1), CRP: C-reactive protein, CVD: Cardiovascular disease, EE: Energy expenditure, GM-CSF: Granulocyte-macrophage colony stimulating factor, HDL: High-density lipoprotein, IL: Interleukin, INF-γ: Interferon gamma, LDL: Low-density lipoprotein, NEFA: Non-esterified fatty acids, SPSS: Statistical Package for the Social Sciences, TC: Total cholesterol, TG: Triglyceride, TNF-α: Tumor necrosis factor alpha, VO_2: Whole body oxygen consumption.

Introduction

Cardiovascular diseases (CVD) are the leading causes of death in US and the world [1], and approximately 610,000 individuals die of CVD in the US each year [2]. Past research has shown that the underlying causes of most cases of CVD are chronic inflammation and elevated blood lipids, and both inflammatory cytokines and lipid mediators are used as biomarkers to assess CVD risks [3]. It is well accepted that positive lifestyle modifications, such as regular engagement in physical activity and high diet quality, leads to health benefits, including improvement in CVD-associated lipid and inflammatory biomarkers. Unfortunately, epidemiological research has repeatedly shown that prevalence of enough physical activity and adequate nutrition among US adults are still far from satisfactory [4,5], suggesting a need of developing other approaches that may serve as an alternative option to reduce the risk of CVD. A potential approach that may warrant further investigation is a systemic exposure to cold stimulus due its potential beneficial physiological and metabolic effects [6,7].

The effects of cold environment on human physiology have been investigated using an acute cold exposure (ACE) paradigm, where study participants were typically exposed to cold environment in a laboratory. A commonly used approach in cold exposure research is to utilize a temperature-controlled room (4-15°C) for 30-120 minutes [7-12]. Alternatively, a liquid-conditioned tube suit perfused with 8°C water for 180 minutes was utilized [6]. Using these experimental approaches, research shows that ACE induces substantial changes in a variety of physiological variables. Some physiological changes are characterized by increases in heat production, oxygen consumption, and energy expenditure, as well as decrease in skin temperature [7-9], whereas other physiological changes include those similar to stress responses, such as increases in epinephrine, norepinephrine, adrenocorticotropic hormone (ACTH), and cortisol [9-12]. Together, data from past research indicate that ACE is a potent environmental stimulus to induce substantial changes in human physiology, some of which resemble metabolic responses to increasing activity levels

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(e.g., increased heat production, oxygen consumption, and energy expenditure). Thus, ACE may result in favorable changes in CVD risk profiles, as well as increases in metabolic rates.

Currently, very little is known whether exposure to cold stimulus brings about positive effects on CVD risks. Cold exposure may depress the immune system and led to increased upper respiratory tract infection or "colds" [13] but research is conflicting [14]. Colder regions and colder winter are generally associated with increased prevalence and severity of CVD, including hypertension, heart attack, and stroke [15] suggesting potentially adverse effects of cold environment on CVD risks. Rodent and equine studies found short and long-term cold exposure increased blood pressure, inflammatory and lipid biomarkers in the blood, and atherosclerosis lesion development [16,17]. Conversely, cold exposure in mice increased brown adipose tissue activity that decreased triglyceride blood concentrations and improved insulin resistance [18] as seen in humans [6]. Human laboratory studies have been conducted examining the effects of ACE on CVD biomarkers. The lab-based studies have measured blood cell counts and a limited number of cytokines in response to ACE [14,17,19] without a consensus as to the effects. Furthermore, the lab studies have failed to address a wide array of inflammatory cytokines, acute phase proteins, and lipid mediators to provide a comprehensive view of CVD risk profiles.

CVD risk is associated with an imbalance between inflammatory cytokines and anti-inflammatory cytokines, hyperlipidemia, and indicators of stress. Blood levels of the lipid biomarkers, such as total cholesterol, low-density lipoprotein (LDL) cholesterol, triglycerides and non-esterified fatty acids (NEFA) are associated with increased CVD risk and are used in the diagnosis of metabolic syndrome [20]. Inflammatory cytokines associated with increased CVD risk include interleukin 1-beta (IL-1β), monocyte chemoattractant protein-1 (MCP-1), IL-4, IL-6, IL-8, granulocyte macrophage-colony stimulating factor (GM-CSF), interferon-gamma (INF-γ), and tumor necrosis factor-alpha (TNF-α) [3,20,21]. High-density lipoprotein (HDL) and anti-inflammatory cytokines IL-2 and IL-10 are associated with a reduced risk of CVD [20,22,23]. In addition, changes in expression of acute phase proteins can be used to assess tissue damage after a CVD event or physiological stress. Chronic inflammation and CVD disease risk has been associated with alteration in the production of particular acute phase proteins including C-reactive protein, ferritin, fibrinogen, procalcitonin, serum Amyloid A, serum Amyloid P, tissue plasminogen, α-2-macroglobin, and haptoglobin [3,20,24-32]. Therefore, to fill the gap in the relevant literature, the present study aimed to examine the effects of ACE on a comprehensive panel of plasma inflammatory, lipid biomarkers and acute phase proteins related to CVD risk.

Materials and methods

Subjects

This study was approved by the Committee for the Protection of Human Subjects in Research of the University of Texas at San Antonio. All data were collected in the Exercise Biochemistry and Metabolism Laboratory, Department of Kinesiology, Health, and Nutrition, the University of Texas at San Antonio. Daily laboratory temperatures range from 22-24°C. Average outdoor temperatures ranged from 18-26°C and participants were not cold acclimated. Body weight and composition were assessed by bioelectrical impedance (Tanita). Twenty individuals gave consent, self-reported as not having thyroid hormone-related disease or to be taking medications affecting metabolism, and then completed the protocol described below. Females were not screened for the phase of the menstrual cycle.

The sample size was estimated based on literature review of relevant research and power analysis. Past studies documenting the effects of ACE on physiological variables were typically conducted with a small number of healthy adults (n=5-16) [6-12] suggesting that the effects of ACE on outcome measures are likely to be large. Therefore, a power analysis was performed, with an alpha = 0.05, a power = 0.80, and a large effect size (f=0.40) [33], and results from the power analysis indicated that 12 individuals would be needed for this study to detect significant effects of ACE on the outcome measures. To account for potential missing data due to subjects' termination of the study protocol and technical issues during the study protocol, a total of 20 individuals participated in this study.

Protocol

Subjects were asked to fast for 10 h, sleep at least 6 h, refrain from caffeine, alcohol, dietary supplements, medications, and any activity (e.g., exercise) above that required for basic grooming and activities of daily living for 12 h prior to their testing session, minimizing inter-subject energy expenditure (EE) variability. Prior to testing, subjects voided their bladders and then sat in a comfortable chair with controlled/minimized visual or auditory stimulation for 10 min followed by instrumentation for the 3.3 h protocol of standard indirect calorimetry. Non-invasive EE estimates were made using indirect calorimetric estimations of human energy utilization using the thermal equivalents of oxygen for the non-protein respiratory quotient (ParvoMedics Inc, Sandy UT). Subjects' movement was kept at a minimum throughout the entire 3.3 h protocol (40 minutes baseline, 30 min ACE, and then 2 h post with extra time for transitions) to control for extraneous body EE. All subjects watched the same non-action movie video with external noise-cancelling headphones to help control stimuli throughout each phase of the protocol, minimizing the possible effects of extraneous stimuli from lab personnel, doors closing, etc. Subjects were seated for the entire protocol. Subjects' tympanic temperature was assessed (ThermoScan 6021 Ear thermometer Braun; Kaz; Hudson, NY, USA). Heart rate was assessed using a heart rate monitor (Polar RS800, Polar, Kempele, Finland).

Pre-ACE

First, subjects submerged their feet and lower legs (up to their knees) in water (27°C) and soaked-towels (27°C) were draped on their thighs, shoulders, arms, and the back of their neck to mimic the prep used during the subsequent cold-exposure phase. Water (27°C) was poured on the subject every minute to maintain temperature during the pre-cold phase. Second, the movie was started, and subjects sat still for the entire 40 min pre-cold period.

ACE

After 40 min of pre-cold data collection, investigators quickly (~30 sec) had subjects move their legs from the 27°C tub into a 7°C water bath. The 27°C towels were replaced by ice water-soaked towels (7°C). A large high-power air fan was positioned towards the subjects. All other instrumentation remained in place during the transition. Water (7°C) was poured on the subjects every minute to maintain temperature during the cold phase. Subjects provided a finger indication of shivering onset and indicated shivering intensity using a Likert-style scale (1=light to 10=severe) when promoted every 5 min throughout the 30 min cold exposure period to report self-reported shivering sensations. Subjects' perceived discomfort was assessed every 5 min and ranged from zero being no discomfort to ten being extreme discomfort to report pain due to ACE. Thirty minutes acute cold exposure has been reported in

the literature [11] and was found to increase resting metabolic rate in previous studies conducted in our lab. Thirty minutes was also chosen to simulate a 30-minute moderate-intensity exercise session that is for physical activity recommendation for the adults.

Post-ACE

After the 30 min cold exposure, investigators quickly had subjects lift their legs from the cold tub into the 27°C water bath. Cold towels were replaced with 27°C towels and the air fan turned off. Subjects sat at quiet rest for 2 h of continued data collection as described above.

Blood and Plasma Collection

Fifteen to thirty milliliters of blood were collected by venipuncture at three-time points: 1) pre-ACE (baseline), 2) at 30 min ACE, and 3) 2 h post-ACE. Blood was collected in tubes containing ethylenediaminetetraacetic acid (EDTA, 24 $mg.ml^{-1}$, pH 7.4) and plasma recovered following centrifugation (15 min at 1,000 x g) and stored at -80°C until subsequent analysis.

Cardiovascular disease biomarkers

Inflammatory cytokines: Ten cytokines associated with cardiovascular disease were measured. Enzyme-linked immune absorbent assay (ELISA) ready-to-go kits (eBiosciences, San Diego, CA) were used to measure interleukin 1beta (IL-1β), and monocyte chemoattractant protein-1 (MCP-1, also known as CCL2) in plasma. The Human Cytokine 8-plex BioPlex Pro assay (Bio-Rad Laboratories, Hercules, CA) was used to measure IL-2, IL-4, IL-6, IL-8, IL-10, granulocyte macrophage-colony stimulating factor (GM-CSF), interferon-gamma (INF-γ), and tumor necrosis factor-alpha (TNF-α) using the Bio-Plex protein array system (Luminex-based technology; Bio-Rad Laboratories, Hercules, CA).

Acute phase proteins: Acute phase proteins associated with cardiovascular disease risk were measured. C-reactive protein (CRP), alpha-2-macroglobin, haptoglobin, serum amyloid P were measured using the Human Acute Phase 4-plex Bioplex Pro assay (BioRad) and ferritin, fibrinogen, procalcitonin, serum amyloid A, and tissue plasminogen activator were measured using the Human Acute Phase 5-plex Bioplex Pro assay (Bio-Rad Laboratories, Hercules, CA).

Lipid biomarkers: Colorimetric assays (Wako Diagnostics, Richmond, VA) were used to measure total cholesterol (TC), low-density lipoprotein cholesterol (LDL), high-density lipoprotein cholesterol (HDL), triglycerides (TG) in plasma, and nonesterified fatty acids (NEFA).

Statistical analysis

The primary aim of this study was to examine the effects of ACE on inflammatory and lipid biomarkers related to cardiovascular disease risk. The sample size was estimated to achieve the primary aim, and cytokine, acute phase protein, lipid biomarker, and physiological data were analyzed with the data from 20 human subjects using repeated measures ANOVA, where the outcome measures were assessed at three time points (pre-ACE, 30 min ACE, and 2 h post-ACE). Additionally, sex differences in the outcome measures were examined as the preliminary analyses using 2 (sex: males and females) × 3 (measurement times: pre-ACE, 30 min ACE, and 2 h post-ACE) mixed-model ANOVA. Post hoc analyses were performed for these analyses using the Least Significant Difference method (SPSS statistical software, IBM) to determine significant differences. Study subject characteristics were analyzed using Student's t-test (SPSS statistical software, IBM). All data are presented as mean ± SEM of 20 human subjects unless stated otherwise. Variables less than 20 resulted from missing participant data or undetectable results. Results were considered statistically significant at the $p<0.05$ level. The analyses with an entire sample were performed to test the primary aims of the study. However, due to sex differences in body composition (reported in Results section below), the analyses on sex differences in the outcome measures were performed to provide supplementary data of interest.

Results

Subject characteristics and physiological parameters

Twenty subjects, with 11 males and 9 females participated this study. Age and BMI were similar between males and females. However, height, weight, lean body mass, fat mass, % body fat, and lean mass normalized to body weight (BW) were significantly different between males and females (Table 1). Physiological parameters measured at pre-ACE (baseline) and at 30 min ACE are summarized in Table 2. Tympanic temperature decreased slightly from pre-cold to after 30 min of ACE (Table 1). However, as expected, heart rate ($p<0.05$), self-reported shivering sensations ($p<0.001$), perceived discomfort ($p<0.001$), VO_2 ($p<0.001$), and EE ($p<0.001$) significantly increased after 30 min ACE than baseline (Table 2). Therefore, these data demonstrate that ACE in the present study resulted in similar metabolic responses to previous studies [34].

Since body composition is significantly different between males and females, sex differences were analyzed and reported in supplementary data (Supplementary Tables 1-4) and combined data are reported in Tables 2-5. As shown in supplementary table 1, there were no sex differences in physiological parameters in response to ACE, except females had significantly higher HR at baseline than males.

Inflammatory Cytokines

The levels of IL-1β, a pro-inflammatory cytokine, significantly increased by 24% at 30 min ACE (p=0.048) and 65% at 2 h post-ACE (p=0.01) compared to baseline (Table 3). The repeated measures

Table 1. Subject characteristics

Variable	Males (n=11)	Females (n=9)	All (n=20)
Age (y)	24.2 ± 0.8	23.6 ± 0.9	23.9 ± 0.6
Height (cm)	175.7 ± 2.8	164.7 ± 3.0*	170.1 ± 2.4
BW (kg)	80 ± 12	67 ± 12*	74 ± 13
BMI (kg/m^2)	26.1 ± 1.1	24.4 ± 0.8	25.4 ± 0
Lean Mass (kg)	69.7 ± 4.1	46.5 ± 3.2*	59.7 ± 4.1
Fat Mass (Kg)	17.1 ± 2.0	11.5 ± 1.5*	13.9 ± 1.4
% Body Fat	14.0 ± 1.4	26.5 ± 1.5*	19.4 ± 2.0
Lean Mass/BW	0.73 ± 0.02	0.86 ± 0.01*	0.81 ± 0.02

Values are expressed as mean ± SEM; BW, body weight; BMI, body mass index; *$p<0.05$, compared to males. n=8 for male and n=6 for female for lean mass, fat mass, and % Body fat

Table 2. Acute cold exposure measured physiological and self-reported parameters

Variable	Baseline	30 min ACE	n
Heart Rate (beats/min)	66.3 ± 2.84	73.9 ± 3.17*	15
Temperature (ºC)	36.8 ± 0.09	36.4 ± 0.11*	20
VO_2 (ml/kg/min)	3.86 ± 0.35	5.78 ± 0.28**	18
EE (kcal/min)	1.38 ± 0.08	2.10 ± 0.09**	18
Shivering Sensations	0.00 ± 0.00	4.05 ± 0.48**	19
Pain	0.00 ± 0.00	1.95 ± 0.59**	19

Values are expressed as mean ± SEM. *$p<0.05$, **$p<0.001$

ANOVA revealed that several cytokines significantly changed in response to ACE when expressed as fold changes from baseline to treatment (Supplementary Figure 1). For example, IL-4 increased by 15% (p=0.018) immediately after cold exposure and increased by 23% from baseline at 2 h post-ACE (p=0.015, Supplementary Figure 1). IL-2 and IL-10, anti-inflammatory cytokines, increased 22% (p=0.025) and 35% (p=0.024) respectively, after 2 h post-ACE (Supplementary Figure 1). However, no significant cold-induced change in the inflammatory cytokines IL-6, IL-8, CCL2, GM-CSF, and TNF-α compared to pre-ACE values (Table 3). With further analysis by sex differences, the levels of IL-2, IL-4, and IL8 were significantly increased in males, but not in females (Supplementary Table 2).

Acute phase proteins

No change was observed at 30 min ACE from baseline measurements for ferritin, fibrinogen, procalcitonin, serum amyloid A, tissue plasminogen, CRP, and haptoglobin (Table 4). The levels of α-2-macroglobulin increased 16% (p=0.005) at 30 min ACE and decreased to pre-ACE levels at 2 h post-ACE (Table 4 and Supplementary Figure 1). These significant changes were seen in males, but not females (Supplementary Table 3). As expected, ferritin, an important protein for storing iron in the tissues, was significantly lower in females compared to males (Supplementary Table 3). Sex differences were present in fibrinogen levels after 30 min ACE compared to baseline, showing a greater increase in females as compared to males.

The repeated measures ANOVA of fold change revealed serum amyloid P increased by 9% (p=0.044) at 30 min ACE and decreased to pre-ACE levels at 2 h post-ACE. Although procalcitonin and CRP did not show a statistically significant difference, there was a trend for increased expression at 30 min ACE (p=0.089 and p=0.065 respectively).

Lipid biomarkers

TC, LDL, TG, and NEFA did not change in response to ACE (Table 5). Interestingly, HDL cholesterol significantly increased at 2 h post ACE by 15% (p=0.006). The increase is largely seen in females (Supplementary Table 4). Females had significantly higher LDL at baseline than males (Supplementary Table 4), along with higher % fat and fat mass (Table 1).

The repeated measures ANOVA of fold change revealed a significant increase in HDL at 30 min ACE by 8% (p=0.032) and increased an additional 5% (p=0.034) at 2 h post-ACE, for a total increase of 13% at 2 h compared to pre-ACE (p=0.002) (Supplementary Figure 1).

Table 3. Measured cytokines in plasma 1) pre-ACE (baseline), 2) after 30min of ACE, and 3) 2h post-ACE

Cytokine (pg/mL)	Baseline	30 min ACE	2 h post ACE	n
IL-1β	18.5 ± 4.33	23.0 ± 4.60**	30.5 ± 4.01**	11
IL-2	5.11 ± 0.380	5.74 ± 0.593	6.16 ± 0.663	20
IL-4	1.28 ± 0.079	1.45 ± 0.050	1.59 ± 0.151	20
IL-6	6.71 ± 0.230	7.01 ± 0.308	7.87 ± 0.623	20
IL-8	6.58 ± 0.391	6.78 ± 0.383	7.04 ± 0.607	20
IL-10	6.33 ± 0.353	7.05 ± 0.683	8.48 ± 1.05	20
CCL2	116 ± 16.5	132 ± 24.2	138 ± 27.7	16
TNFα	18.7 ± 1.30	18.4 ± 1.45	22.2 ± 2.82	20
GMCSF	18.0 ± 3.28	14.5 ± 1.79	23.4 ± 3.89	12

Values are expressed as mean concentration ± SEM; *p<0.05, **p<0.01 compared to baseline.

Table 4. Measured acute phase proteins in plasma 1) pre-ACE (baseline), 2) after 30min of ACE, and 3) 2h post-ACE

Acute Phase Proteins	Baseline	30 min ACE	2 h post ACE	n
Ferritin (ng/mL)	19.3 ± 3.55	19.6 ± 3.97	19.4 ± 3.91	20
Fibrinogen (mg/dL)	9.82 ± 0.500	10.0 ± 0.647	10.3 ± 0.942	20
Procalcitonin (ng/mL)	0.512 ± 0.076	0.520 ± 0.079	0.476 ± 0.056	20
Serum Amyloid A (mg/L)	0.364 ± 0.051	0.337 ± 0.044	0.361 ± 0.048	20
Tissue Plasminogen (ng/mL)	1.38 ± 0.124	1.32 ± 0.090	1.30 ± 0.093	20
C-reactive protein (mg/L)	0.048 ± 0.016	0.051 ± 0.016	0.049 ± 0.016	20
α-2-macroglobin (mg/dL)	127 ± 7.57	147 ± 9.63**	129 ± 11.0	20
Serum Amyloid P (mg/dL)	2.35 ± 0.169	2.46 ± 0.176	2.44 ± 0.192	20
Haptoglobin (mg/dL)	26.3 ± 5.54	26.6 ± 5.63	26.8 ± 4.99	16

Values are expressed as mean concentration ± SEM; *p<0.05, **p<0.01

Table 5. Measured lipids in plasma 1) pre-ACE (baseline), 2) after 30min of ACE, and 3) 2h post-ACE

Lipid biomarkers	Baseline	30 min ACE	2 h Post ACE	n
Triglycerides (mg/dL)	57.4 ± 7.39	61.4 ± 8.59	65.6 ± 11.2	16
Free Fatty Acids (uM)	417 ± 68.6	412 ± 47.7	437± 54.0	15
Total Cholesterol (mg/dL)	122 ± 6.44	126 ± 7.20	122 6.66	17
LDL (mg/dL)	113 ± 6.94	119 ± 8.93	114 ± 7.27	15
HDL (mg/dL)	38.1 ± 1.96	41.7 ± 2.51	43.7 ± 2.57**	12

Values are expressed as mean concentration ± SEM; *p<0.05, **p<0.01

Discussion

In this study, the relationship between ACE and inflammatory and lipid biomarkers that are associated with CVD before, at 30 min ACE and 2 h post-ACE was investigated. This is the first comprehensive study to measure 5 lipid biomarkers, 10 cytokines, and 9 acute phase proteins at multiple time points on the same individual allowing for the statistical power to detect within-person change. Significant increases in heart rate, self-reported shivering sensations, perceived discomfort, VO_2 and EE occurred after 30 min ACE. IL-1β, HDL, and α-2-macroglobin significantly increased, while fold change analysis detected additional significant differences in IL-2, IL-4, IL-10, and serum amyloid P following ACE.

Predictively, self-reported shivering sensations, whole body oxygen consumption, and EE increased in response to ACE as previously reported [34]. Conflicting data exists on the effect of acute cold exposure on heart rate where cold-water immersion increases [35] and cold air exposure decreases [36] heart rate after 2 h ACE. In this study, heart rate significantly increased, but largely due to an increase in heart rate in females. Increased heart rate due to ACE in females as compared to males has previously been reported [36]. Additionally, the present study indicated sex differences in self-reported discomfort due to ACE, with females rating cold discomfort higher than males. Given that the participants in this study were exposed to cold water at 7°C during ACE, which is within a range of water temperatures that are found to be cold enough to produce cold pain sensation [37], the findings are generally in agreement with past research reporting that women are more sensitive to pain stimuli compared to men [38]. Sex difference in long-term cold acute exposure is attributed largely to differences in body mass, fat mass, surface area, and hormones [34].

The changes in three out of the four cytokines post-ACE have been previously investigated in the literature [17,39,40]. IL-1β is a pro-inflammatory cytokine secreted by monocytes and its expression is highly associated with chronic inflammatory disease including CVD. Previously, no change in IL-β due to ACE in a temperature-controlled room have been reported [17], while our study reported an increase at 30 min ACE with further increase at 2 h post ACE. IL-4

is another pro-inflammatory cytokine that increased at 30 min ACE and 2 h post-ACE in the current study. IL-4 has been implicated in atherosclerosis development, although the exact mechanism in inflammatory processes related to CVD is unclear [21]. Increases in IL-4 have been reported in cold exposure greater than 3h in animals [19,22]. It is unclear how increases in IL-1β and IL-4 play a role in disease risk in ACE, but the acute elevation indicates activation of the immune system in response to ACE. Conversely, IL-2 and IL-10, two cytokines that signal the resolution of inflammation and are involved in anti-inflammatory responses [22,23] increased 2 h post-ACE in our study. In agreement with our study, a previous study shows elevated IL-2 and IL-10 in response to ACE in a climate-controlled chamber with or without exercise [19]. Taken together, it appears that ACE triggers specific cytokine release, temporally modulating inflammatory and anti-inflammatory processes.

Immune differences in males and females are well documented and are dictated primarily by genes on the sex chromosome and hormones [41,42]. Typically, females have a stronger immune response to infection which correlates to increased survival and decreased susceptibility to infection than males [41]. In this study, males had an increased immune response due to ACE at 2 h for Il-2, IL-4, and IL-8 compared to baseline. Females had increased expression of these cytokines, but levels did not reach statistical significance. This may be due to fluctuations of cytokines during specific phases of the menstrual cycle [42], which was not accounted for in this study, or the small number of females.

Seven out of the nine acute phase proteins measured did not change in response to ACE. Significant increases in α-2-macroglobulin and serum amyloid P were measured at 30 min ACE but returned to pre-ACE after 2 h post-ACE. Both acute phase proteins have not been previously studied in response to ACE. Alpha-2-macroglobulin is a protein involved in humoral defense but has been investigated as a marker for CVD, but the data is conflicting [26,28]. Elevated serum amyloid P in blood is associated with increased angina and myocardial infarction [31] and may play a role in cardiac remodeling [27]. In summary, acute phase proteins remained unchanged or returned to pre-ACE values 2 h post-ACE. Based on these results, α-2-macroglobulin, serum amyloid P, and the seven other acute phase proteins may not have a large role in acute responses to ACE.

Sex differences of the acute phase proteins were found between ferritin, fibrinogen, CRP, and α-2-macroglobulin. Ferritin levels in females was lower than males at each time point, which is consistent with data showing menstruating females have lower levels of iron [43]. Surprisingly, the average ferritin values indicate below normal values of 12-150 ng/mL for females [44], indicating the female study population may be at risk for iron-deficiency anemia. Increases in α-2-macroglobulin in males at 30 min ACE was likely due to the lower baseline values as compared to females, although both males and females increased at 30 min ACE and returned to baseline values at 2 h post ACE. Both fibrinogen after 30 min ACE and CRP at 2 h post ACE increased in females only. Elevations in acute phase proteins, fibrinogen [20] and CRP [25] among females indicates potential CVD risk due to ACE.

Dyslipidemia, characterized by elevated TC, LDL, TG, or NEFA and decreased HDL, is associated increased risk for CVD. Previous studies have documented an increase in plasma NEFA and TG in response to ACE to provide substrate for uncoupled oxidative phosphorylation (non-shivering thermogenesis) in brown adipose tissue. In this study, plasma NEFA and TG did not significantly increase in response to ACE. The thermogenic effect of shivering likely prevented a large decrease tympanic temperature during this short-term exposure. The duration of ACE may be important as other studies reported increases of NEFA or TG after ≥ 90 min cold exposure [45,46] that may induce non-shivering thermogenesis. Interestingly, HDL incrementally increased by 8% with 30 min ACE and an additional 8% 2 h post-ACE which is consistent with reported data in cold exposure in a temperature-controlled room for 60 min in humans [17] and 30 min in rats [46]. Taken together, these data suggest ACE may have a positive acute effect on HDL blood levels. Future studies are warranted to investigate whether frequent exposure of ACE could elevate HDL levels.

Sex differences in lipoprotein metabolism and plasma profiles are well documented [47]. Typically, premenopausal females have lower plasma TC, TG and LDL and higher HDL cholesterol levels than males. In this study, female HDL levels increased due to ACE and remained elevated at 2 h. Surprisingly, LDL baseline values were higher in females than males but differences in TC, TG, FFA, and HDL were not found between males and females, likely due to the low power and high variation between individuals.

Limitations of this study include the homogenous population of convenience consisting of young, healthy adults, lack of core or skin temperature, lack of control group for venipuncture stress and non-cold controls, and females were not screened for stages of the menstrual cycle or oral contraception use. It is likely that ACE may result in different expression of these biomarkers depending on age, body mass, and disease status. For example, individuals with chronic inflammation and elevated levels of cytokines, acute phase proteins, and lipids may respond differently to ACE due to the fact their immune system is already activated, and lipid metabolism is altered. Supporting our assumption, we found that interferon gamma (INF-γ), one of pro-inflammatory cytokines, was undetectable in this healthy population (data not shown). Although this study lacks controls for cold exposure and venipuncture stress, each study participant is their own control at baseline that represents a "non-cold stressed" control and venipunctures were a constant stress and unlikely to affect differences reported from baseline, 30 min ACE, and 2 h post ACE. Core temperature can be assumed unchanged based on the small decreases in tympanic temperature but was not directly measured. Collecting skin temperatures was not possible with our study protocol due to continuous water pouring. Previous reports suggest tympanic and skin temperatures do not differ during 120 minutes of cold exposure and tympanic temperatures correlate with drops in skin temperature [48]. A drop in 1°C of tympanic temperature resulted in physiological responses to acute cold (elevation in TSH) [49]. Thus, tympanic temperatures can be used to detect physiologically relevant drops in temperature in response to acute cold that may mirror skin temperatures. Lastly, females respond differently to cold exposure when taking oral contraceptive and in different stages of the menstrual cycle [34] which was not accounted for in this study.

Conclusions

In conclusion, our results provide data on the effect of ACE on inflammation, the acute phase response, and lipid biomarkers. Acute cold exposure has the potential to induce pro-inflammatory cytokines associated with CVD (IL-1β, IL-4), but also increased anti-inflammatory cytokines (IL-2 and IL-10) which can dampen the inflammatory response. Increased HDL levels with increased EE and VO2 are the positive effects of ACE. However, ACE could give the negative impact on CVD, particularly seen in females (i.e., elevated

levels of fibrinogen and CRP); thus, the long-term risk of CVD needs further exploration to assess the risks or benefits of ACE. Future studies with diverse subjects varying in age and body composition and studies aimed to determine the effectiveness of ACE as compared to diet and exercise are warranted.

Compliance with Ethical Standards

The authors declare that they have no conflict of interest.

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Informed consent was obtained from all individual participants included in the study.

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