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Ischemia Reperfusion Unveils Impaired Capacity of Older Adults to Restrain Oxidative Insult

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Abstract

Age independently predicts poor outcome in a variety of medical settings including sepsis, trauma, severe burns, and surgery. Since these conditions are associated with oxidative stress, we hypothesized that the capacity to constrain oxidative insult diminishes with age, leading to more extensive oxidative damage during trauma. To test this hypothesis, we used supra-systolic inflation of an arm blood pressure cuff to safely induce localized forearm ischemia/reperfusion (I/R) and quantified plasma F₂-isoprostane (IsoP) levels in serial blood samples. Prior to I/R, IsoP levels were similar in young (20–33 yrs) and older adults (62–81 yrs). After I/R challenge, the magnitude and duration of increased IsoP levels was significantly greater in older adults. Because aging is associated with declining levels of sex hormones that contribute to regulation of antioxidant enzyme expression, we then examined the response to I/R in older women receiving hormone replacement therapy, and found these women did not manifest the amplified IsoP response found in untreated older women. These findings demonstrate that aging impairs the ability to restrain oxidative damage after an acute insult, which may contribute to the increased vulnerability of older adults to traumatic conditions, and establishes a useful method to identify effective interventions to ameliorate this deficiency.

Introduction

Older adults are more vulnerable to a variety of serious medical conditions including sepsis, myocardial infarction, traumatic injury, burns, and surgical procedures such as coronary artery bypass and organ transplantation [1–5]. While the greater vulnerability of older adults has been a formidable dilemma in geriatric medicine, the basis of this vulnerability has not been fully established. These medical conditions are also associated with substantially enhanced levels of reactive oxygen species (ROS) [6–11]. We hypothesized that individuals significantly vary in their capacity to resist oxidative stress during traumatic conditions, with older adults being significantly less able to restrain oxidative stress than young adults. If this hypothesis is correct,

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then the poorer clinical outcomes in older adults would likely result from a greater oxidative injury incurred during exposure to traumatic conditions.

Consistent with the notion that increased oxidative damage correlates with poorer clinical outcome, we recently reported that for severely-ill older patients (≥ 70 yrs) plasma levels of F₂-isoprostanes (IsoPs) strongly correlated with their APACHE II score (an objective measure that predicts severity of illness and mortality [12].) IsoPs were chosen as the primary biomarker of acute oxidative stress for the study based on a comprehensive, multi-institution study coordinated by NIEHS comparing a variety of widely-used plasma and urinary biomarkers of oxidative stress that found that the IsoPs substantially outperformed other biomarkers both in terms of sensitivity and reliability [13]. The greater sensitivity of IsoP levels to oxidants compared with other biomarkers may arise from the relatively facile release of IsoPs formed in tissue out into plasma.

Whether older adults differ from young adults in their ability to constrain an oxidative insult has not been established. Several large cross-sectional studies in human populations found that mean levels of IsoPs did not differ in young versus older adults in the absence of acute conditions [14-16]. However, measurements under resting conditions do not necessarily predict the individual's capacity to limit oxidative damage during acute conditions, which requires adaptive responses including downregulation of ROS producing pathways and/or upregulation of antioxidant enzyme defenses.

Differential survival after an acute oxidative insult has been described in the model organism *C. elegans*. In these studies, young worms survived an acute oxidative insult by upregulating antioxidant enzymes, but old worms failed to adapt and lost viability [17]. Whether older and younger human adults also differ in their ability to defend against oxidative insults is unclear. Studies attempting to measure changes in oxidative resistance with age using *ex vivo* oxidation of plasma have been ambiguous [16,18]. Importantly, oxidizing plasma *ex vivo* does not provide an assessment of an individual's complete capacity to constrain an oxidative insult because it does not capture the adaptive responses involving downregulation of ROS producing pathways and/or upregulation of the expression of antioxidant enzyme defenses. Furthermore, although ascorbate is the primary antioxidant that protects plasma against lipid peroxidation and is the first antioxidant to be depleted when plasma is exposed to ROS [19,20], *in vivo* exposure to low levels of ROS (1 ppm ozone) does not significantly alter plasma ascorbate levels even while significantly depleting tissue ascorbate levels and increasing IsoP levels [21]. Therefore, there is not necessarily a direct relationship between the extent of plasma resistance to oxidation (which is principally modulated by its small molecule antioxidants content) and the extent of tissue/organ resistance to oxidation which is modulated both by tissue levels of small molecule antioxidants and the level of expression of antioxidant enzymes.

Ischemia/reperfusion (I/R) elicits ROS formation and is an element of many acute traumas [22,23]. Forearm I/R induced by suprasystolic inflation of a blood pressure cuff has been safely used to examine the effect of I/R on endothelial function *in vivo* [24,25]. We therefore utilized forearm I/R to determine whether young and older adults significantly differ in their extent of oxidative stress after this oxidative insult. Because sex hormones have been implicated in inducing antioxidant defenses [26-33] and their levels decline with age in both men and women, we also examined whether older women receiving hormone replacement therapy would differ from untreated women in their response.

Materials and Methods

Enrollment of subjects

For the primary study, we recruited 20 healthy young adults (10 men, 10 women; ages 20-33, mean age 25 ± 4 y) and 20 healthy older adults (11 men, 9 women ages 62-81, mean age 70 ± 6 y) who gave informed, signed consent (Table 1). Health of individuals was established both by self-report and by brief physical exam. Exclusion criteria included apparent disease, smoking, uncontrolled hypertension, BMI $> 30 \text{ kg/m}^2$, pregnancy, hormone replacement therapy, and intake of antioxidant supplements in excess of the equivalent of a single daily dose of a multi-vitamin. Eight additional women (age 66 ± 6 y) were recruited who met the inclusion criteria for the primary study except that they were receiving some form of hormone replacement therapy (HRT) that included estrogens. The specific estrogen therapy varied by subject. Two women were receiving estrogen patches and one woman each was receiving Premarin, oral estrogen, Estratest HS, Prempro, Menest (pellet), or estradiol+progesterone. Estrogen levels were not measured. A total of 5 young and 4 older subjects were studied at Vanderbilt, and 15 young and 24 older subjects (16 without HRT and 8 with HRT) were studied at the Kronos Longevity Research Institute (KLRI). The Institutional Review Board for Vanderbilt University and the Western IRB for the KLRI approved the studies conducted at their respective institutions.

Induction of ischemia/reperfusion

Participants came to the Vanderbilt General Clinical Research Center in Nashville, TN or to the KLRI Clinical Research Center in Phoenix, AZ on the morning of the study. An intravenous catheter was placed into an antecubital vein on the arm and 10 ml of blood was drawn. A blood pressure cuff was then placed on the upper arm of the same arm, and the cuff inflated to 200 mm Hg for three periods of 10 minutes separated by 2 minutes with the cuff deflated. An additional 10 ml of blood was drawn at 15, 30, 60, 120, 180, and 240 minutes after the final cuff deflation. The procedure was well tolerated by all subjects.

Biochemical Measurements

The blood samples were centrifuged, plasma extracted, and frozen at -70°C until analysis. All samples were analyzed at Vanderbilt University with samples from KLRI being shipped on dry ice. Free IsoPs in plasma were quantified by gas chromatography/negative ion chemical ionization-mass spectrometry with $[^2\text{H}]8\text{-iso-PGF}_{2\alpha}$ as an internal standard [34]. The integrated area under the response curve (AURC) for 60, 120, 180, and 240 min were calculated by the method of the trapezoidal rule[35] with the pre-I/R value representing zero. Ascorbate levels in plasma were measured by ion pair HPLC and electrochemical detection as previously described [36].

Statistical analysis

Analyses of study results focused on estimating the association between the IsoP levels and age groups. Repeated Measures ANOVA as well as the General Linear Model (ANOVA) were applied to examine the difference between the age groups across all time points and at each time point respectively. The adjustments of gender effect and the interaction effect between age and gender were employed in the General Linear Model; however, due to the limited sample size the multiple comparison procedure was not applied. Similar results were found in the non-parametric tests. No significant site-specific effects were found. All tests were two-sided with a significance level of 0.05. Data analyses were performed using R (version 2.7.2) and GraphPad Prism (version 4.03). Data are presented as means $\pm$ SEM except for Table 1 where the data are presented as means $\pm$ SD.

Results

Twenty minutes of suprasystolic inflation of a blood pressure cuff has previously been used to induce mild forearm I/R [24,25]. Preliminary investigations in our laboratory found that this 20 min I/R protocol also invoked a temporary rise in IsoP levels, but that a protocol using three 10 min periods of suprasystolic cuff inflation separated by two min deflated periods invoked a somewhat greater IsoP response and was more comfortably tolerated (data not shown). We therefore used the 3 × 10 min I/R protocol to determine the response in 20 healthy young adults and 20 healthy older adults. Prior to I/R, plasma IsoP levels did not differ significantly between the young (39.6 ± 3.3 pg IsoP ml⁻¹) and older adults (34.4 ± 4.2 pg IsoP ml⁻¹, Student's t-test $p = 0.34$). Plasma ascorbate levels also did not significantly differ between young (68.2 ± 6.2 μM) and older adults (64.8 ± 7.4 μM, $p = 0.89$). In response to I/R, however, IsoP levels increased to a significantly greater extent in the older adults and remained elevated for a more prolonged period of time than in young adults (Figure 1A). Unlike young adults, where IsoP levels returned to pre-I/R baseline levels or lower by 240 min after I/R, IsoP levels in older adults remained elevated above baseline throughout the four hour time period. To analyze the differences in integrated responses between the two groups, we calculated the area under the response curve (AURC) for each individual through various collection times. AURC values differed significantly between young and older adults when integrated through 60 min or longer (Figure 1B). AURC values did not significantly correlate with BMI. Consistent with lipid peroxidation occurring primarily in muscle tissue rather than the plasma compartment, there were no significant changes in plasma ascorbate levels of either age group at 30 min (young 67.0 ± 5.2 μM; older 63.1 ± 9.8 μM) or 240 min post-I/R (young 60.3 ± 4.8 μM; older 59.6 ± 7.4 μM).

A number of studies have implicated estrogens as potentially protective against oxidative damage [27-32]. To explore the impact of estrogen levels on the capacity to constrain oxidative stress, we recruited a second group of older women using the same criteria as in our primary study, except that these women were required to be receiving hormone replacement therapy (HRT, mean duration 18.4 ± 3.9 y.) This group of older women had a significantly lower IsoP response to I/R than the older women subgroup in the primary study (non-HRT) (Figure 2).

Discussion

Although age is well documented as an important risk factor for poor outcomes in a variety of acute medical interventions and illnesses such as sepsis and major surgical procedures (1-5), the underlying basis for this risk remains to be fully elucidated. A previous study in the model organism *C. elegans* revealed a loss of adaptability in old worms to oxidative stress as monitored in terms of induction of superoxide dismutase [17]. We have recently expanded these studies in *C. elegans* using genome-wide microarray analyses and RNAi studies and discovered an extensive number of genes that are differentially expressed in old and young worms in response to the oxidant juglone of which many were regulated by the FOXO homologue daf-16 and the Nrf2 homologue SKN-1[37]. These pathways regulate a large number of antioxidant genes, and overexpression of daf-16 significantly increases nematode life-span[38-40]. However, it has not been established whether humans also undergo similar age-related changes in their ability to mount an integrated adaptive response to acute oxidative stress.

This study revealed fundamental age-related differences in the ability of adult humans to restrain oxidative stress induced by a relevant oxidative challenge. These differences were not apparent in the absence of the I/R challenge, but manifested early after I/R (15 min) and were sustained throughout the entire study period (240 min). Identification of this difference provides a plausible mechanism for the well documented increased vulnerability of older adults

during a variety of acute medical interventions and illnesses, namely that failure to constrain the oxidative injury associated with these conditions leads to greater organ damage and dysfunction. Our additional finding that older women receiving HRT did not manifest the amplified oxidative stress after I/R suggests that female sex hormones can play an important role in the defense against oxidative insults in females.

Two processes may be invoked to explain our findings: (a) differences in constitutive antioxidant defenses and/or (b) differences in the ability to upregulate antioxidant defenses and/or downregulate ROS forming pathways. The latter is more likely because resting levels of IsoPs and ascorbate levels were not different between young and older individuals. A robust upregulation of antioxidant enzymes within 15 min after exposure to oxidative stress has been demonstrated in humans, so that an impaired ability to upregulate these enzymes in older adults seems likely [41]. Our finding with women receiving HRT is also consistent with upregulation of antioxidant enzymes, as estrogens can upregulate the Nrf2 pathway [42] and estrogens protect against oxidative injury through both estrogen receptor-dependent and independent mechanisms [27,28,31,32].

The use of HRT has been a subject of significant controversy since the original report of Women's Health Initiative (WHI) study[43]. Although the initial analysis indicated a significant increase in cardiovascular risk with HRT, this study has been criticized because the majority of women commenced therapy more than 10 years after menopause. Estrogen may delay onset of the early stages of atherosclerosis, but trigger events in existing atherosclerotic lesions [44]. Recent secondary analysis of the WHI study found that woman who began HRT within 10 years of menopause tended to have a slightly decreased risk for cardiovascular heart disease, while those that began more than 20 years after menopause had a significantly increased risk [45]. All of the women in our HRT group had begun receiving HRT within 10 years of menopause. Our observation that the HRT group had a significantly greater capacity to limit oxidative injury than the untreated group provides a plausible mechanism for the postulated beneficial effect of estrogen in early stages of atherosclerosis. It also provides a plausible mechanism for the improved outcome seen with HRT in mouse models of acute traumatic injury [46]. Additional studies will be needed to elucidate the precise mechanisms whereby aging and sex hormones exert their effects on the adaptive response in humans.

Additional studies will also be needed to determine whether the extent of oxidative stress after forearm I/R is a clinically useful indicator of an individual's general susceptibility to an oxidative insult. For instance, a recent study demonstrated the value of implementing stringent interventional measures to reduce inflammation and oxidative stress for potentially high-risk patients undergoing cardiopulmonary bypass surgery [47]. The forearm I/R test might be useful as a means for accurately identifying patients at risk for excessive oxidative injury for whom stringent interventional measures would be appropriate.

The forearm I/R test may also provide a valuable means to assess the clinical efficacy of potential antioxidant pharmacological interventions. This is important because significant reductions in the level of oxidative stress biomarkers using antioxidants, at least for vitamin E and ascorbate, only consistently occurs in human subjects that already have elevated levels of these biomarkers and not for subjects with normal basal levels [48]. Therefore the clinical pharmacology of antioxidants and particularly the doses needed to suppress oxidative stress in humans for most antioxidants remain to be defined. The clinical importance of such information was recently highlighted in regards to interpretation of clinical trials of vitamin E for the prevention of cardiovascular disease [49,50]. By inducing temporary elevations in oxidative stress, our forearm I/R method may provide a straight forward means to determine the effective dose of antioxidants for use in clinical trials.

Conclusions

The capacity to constrain oxidative insults is manifestly diminished in older adults. This diminished capacity to constrain oxidative stress provides a plausible mechanism for the poor outcomes associated with older age in a variety of medical conditions. Our method of identifying this diminished capacity, mild forearm I/R, may also provide the means to identify at-risk patients and appropriate interventions to improve outcomes.

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List of Abbreviations

F₂-isoprostanes, IsoPs; hormone replacement therapy, HRT; ischemia/reperfusion, I/R.

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FIGURE 1.

Comparison of oxidative stress in young and older adults induced by forearm I/R.

(a) 3×10 minute forearm I/R increased forearm plasma IsoP levels in older adults (filled squares, $n = 20$, values are mean $\pm$ SEM) to a greater extent than young adults (open squares, $n = 20$, repeated measures ANOVA, post I/R time, $p < 0.001$, age group $p = 0.020$).

(b) Analysis of the IsoP response to I/R when calculated as integrated area under the response curve (AURC) for 60, 120, 180, and 240 min. Values are mean $\pm$ SEM. The p-values shown for each time point were calculated using the ANOVA model to determine the gender-adjusted age effect.

FIGURE 2.

The effect of hormone replacement therapy (HRT) on the response to I/R of older women. The IsoP response (240 min AURC) of the older women subgroup in the primary study (non-HRT, n=9) was significantly higher than a second group of older women recruited by the same criteria except that they were receiving HRT (HRT, n=8). The response of the HRT group did not significantly differ from the young women (YW), young men (YM), or older men (OM) subgroups of the primary study. (HRT vs non-HRT ($p=0.02$), HRT vs YW ($p=0.99$), HRT vs YM ($p=0.79$), HRT vs OM ($p=0.31$)).

Table 1

SUBJECT CHARACTERISTICS

	All	Young		All	Older		HRT-group
		Men	Women		Men	Women	
N	20	10	10	20	11	9	8
Age (yrs)	25 ± 4	24 ± 4	26 ± 4	70 ± 6 ^a	68 ± 6	73 ± 6	66 ± 6 ^b
Height (cm)	171 ± 10	177 ± 9	165 ± 8	173 ± 11	182 ± 7	164 ± 4	169 ± 5 ^b
Weight (kg)	69.0 ± 12.6	75.4 ± 10.4	62.6 ± 11.7	78.1 ± 15.7	87.9 ± 14.4	67.1 ± 8.1	66.6 ± 10.3
BMI (kg/m ²)	23 ± 3	24 ± 3	23 ± 3	26 ± 3 ^a	27 ± 3	25 ± 2	23 ± 3
SBP (mm/Hg)	113 ± 12	118 ± 9	108 ± 14	118 ± 11	119 ± 11	118 ± 11	115 ± 11
DBP (mm/Hg)	70 ± 7	71 ± 8	69 ± 6	75 ± 8 ^a	77 ± 7	72 ± 8	72 ± 7

Data presented as means ± SD. BMI = Body Mass Index, SBP = Systolic Blood Pressure, DBP = Diastolic Blood Pressure.

^a significantly different from young *P* <0.05

^b significantly different from older women *P* <0.05