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# Endothelial Cell Fatty Acid Unsaturation Mediates Cold-Induced Oxidative Stress

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**Abstract** Ultraprofound hypothermia (<5°C) induces changes to cell membranes such as liquid-to-gel lipid transitions and oxidative stress that have a negative effect on membrane function and cell survival. We hypothesized that fatty acid substitution of endothelial cell lipids and alterations in their unsaturation would modify cell survival at 0°C, a temperature commonly used during storage and transportation of isolated cells or tissues and organs used in transplantation. Confluent bovine aortic endothelial cells were treated with 18-carbon fatty acids (C18:0, C18:1n-9, C18:2n-6, or C18:3n-3), C20:5n-3 or C22:6n-3 (DHA), and then stored at 0°C without fatty acid supplements. Storage of control cells caused the release of lactate dehydrogenase (LDH) and a threefold increase in lipid peroxidation (LPO) when compared to control cells not exposed to cold. Pre-treating cells with C18:0 decreased the unsaturation of cell lipids and reduced LDH release at 0°C by 50%, but all mono- or poly-unsaturated fatty acids increased injury in a concentration-dependent manner and as the extent of fatty acid unsaturation increased. DHA-treatment increased cell fatty acid unsaturation and caused maximal injury at 0°C, which was prevented by lipophilic antioxidants BHT or vitamin E, the iron chelator deferoxamine, and to a lesser extent by vitamin C. Furthermore, the cold-induced increase in LPO was reduced by C18:0, vitamin E, or DFO but enhanced by DHA. In conclusion, the findings implicate iron catalyzed free radicals and LPO as a predominant mechanism of endothelial cell injury at 0°C, which may be reduced by increasing lipid saturation or treating cells with antioxidants. J. Cell. Biochem. 99: 784–796, 2006. 

**Key words:** hypothermia; lipid peroxidation; endothelial cells; stearic acid; poly-unsaturated fatty acid

Hypothermia is widely used in laboratory and clinical practice to protect isolated cells, tissues and organs from injury during hypoxic or ischemic conditions. The protective temperatures employed are largely determined by practical constraints and the clinical or research application of the hypothermia. For example, mild hypothermia (32–34°C) is used to treat patients following cardiac arrest [Dae et al., 2002; Holzer, 2002; Holzer et al., 2005] or stroke [Schwab et al., 1998; Bernard and Buist, 2003; Thomé et al., 2005] to improve their cardiac or neurological recovery, while moderate-to-deep hypothermia (32–15°C) is used during procedures such as coronary artery bypass surgery [Hayashida et al., 1994; Vazquez-Jimenez et al., 2001; Allen et al., 2003] or aneurysm repair surgery [King et al., 1998; Moriyama et al., 2001; von Segesser et al., 2001] to reduce ischemic injury to the heart or brain. Significantly lower temperatures (0–4°C) are required for the transportation and storage of organs or tissues used in transplantation or during handling of isolated cells and tissues in the research lab. At these temperatures, however, the hypothermia per se initiates metabolic, structural and functional injury that limits the storage time and clinical utility of grafts. For some transplant organs such as the heart, the cold ischemia is limited to about 4 h or less [Young et al., 1994; Hosenpud et al., 2001] while allograft tissues such as heart valves or arteries are stored for several days [Yacoub et al., 1995; Chiesa et al., 1998; Crowe et al., 1998; Gall et al., 1998; Pukacki et al., 2000]. Better preservation methods are required to

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reduce cold-induced injury and allow for extended storage times without compromising cell viability or tissue function.

Hypothermic injury is attributed to several mechanisms, including the loss of ionic homeostasis [Martin et al., 1972; Southard and Belzer, 1980], the uncoupling of cellular metabolism [Hochachka, 1986], the transition of membrane lipids from liquid to gel phase [Kruuv et al., 1983; Drobnis et al., 1993] and oxidative stress [Rauen et al., 1997; Camara et al., 2004]. Membrane lipids may play an important role in these mechanisms. Cooling decreases membrane fluidity and ultimately brings on a change in lipid phase that may alter the barrier properties of the membrane [Quin, 1985; Drobnis et al., 1993; Hansen, 1993; Lande et al., 1995] and the function of membrane-associated proteins such as ion pumps [Silvius and McElhaney, 1980], transporters [Thilo et al., 1977], or enzymes [Livingstone and Schachter, 1980]. In mammalian cells, membrane fragility and cell sensitivity to cold shock correlate with lipid phase transitions that occur at relatively high hypothermic temperatures [Drobnis et al., 1993; Hansen, 1993]. Protection from lipid transitions and increased low temperature survival has been achieved by treating cells with membrane fluidizers [Kruuv et al., 1983]. Polyunsaturated fatty acids (PUFA) also improve membrane function during cooling by increasing membrane fluidity. In nature, fish and other poikilotherms have the ability to restructure their membranes with PUFA to maintain important functional properties as temperatures decrease [Hazel, 1984]. Similarly, fish that inhabit permanent cold climates have membranes that are adapted to cold, partly by increased membrane PUFA content [Abele and Puntarulo, 2004]. Thus, increasing the PUFA content of mammalian cells has the potential to increase membrane fluidity and function at low temperatures and improve cell survival during hypothermia.

An increase in the PUFA content of cells also has the potential to increase the susceptibility of their membranes to lipid peroxidation (LPO) reactions and membrane damage during hypothermia. Hypothermia increases oxidative stress in the cell [Rauen et al., 1997; Camara et al., 2004], and increased levels of intracellular catalytic iron during hypothermia may play a role in this damaging process [Zieger et al., 1990; Rauen et al., 2000; Huang and Salahudeen, 2002]. Cold-induced LPO and its augmentation by iron in cold-perfused (oxygenated) or cold-ischemic hearts [Menasché et al., 1990; Ely et al., 1992; Magni et al., 1993; Dobsak et al., 2002], cold-ischemic kidneys [Fuller et al., 1986; Green et al., 1986; Healing et al., 1989] and the cooled rabbit leg model [Iyengar et al., 1990] have been reported. Oxidative injury to the vascular endothelium is a key event in several pathological conditions such as ischemia-reperfusion injury [Tsao et al., 1990; Seccombe et al., 1994], inflammation [Calder, 2002], cardiac allograft vasculopathy [Day et al., 1995], and atherosclerosis [Davignon and Ganz, 2004].

We hypothesized that fatty acid substitution of endothelial cell lipids and alterations in their unsaturation would modify cell survival at 0°C, a temperature commonly used during storage and transportation of isolated cells or tissues and organs used in transplantation. In this study, the cold-induced release of lactate dehydrogenase (LDH) and LPO was measured in bovine aortic endothelial cells following alterations in cellular lipid unsaturation and subsequent hypothermia. The results of this study demonstrate that increasing endothelial cell fatty acid unsaturation sensitizes cells to hypothermia and increases cold-induced oxidative stress. No benefit was derived from increasing cellular PUFA content.

## MATERIALS AND METHODS

### Cell Culture

Proliferating bovine aortic endothelial cells (passage 2) were obtained from Cell Systems (Kirkland, WA) and maintained at 37°C in an atmosphere of 95% air + 5% carbon dioxide using Dulbecco's minimum essential medium (DMEM) supplemented with nonessential amino acids, antibiotic-antimycotic solution (penicillin G, 20 units/ml, streptomycin sulfate, 20 μg/ml; all from Gibco, Grand Island, NY), endothelial cell growth supplement (Upstate Biotechnology, Lake Placid, NY), and 10% fetal bovine serum (Hyclone, Logan, UT). This medium will henceforth be referred to as growth medium. In preparation for hypothermia experiments, cells (passages 3–7) were washed with phosphate-buffered saline (PBS, from Gibco), trypsinized (0.1% in ethylenediaminetetraacetic acid, Gibco), resuspended in growth medium and an aliquot was mixed with trypan blue and counted using a hemocytometer. Cells were

then seeded into culture flasks at a density of $8 \times 10^3$ viable cells/cm$^2$ (12.5 or 75 cm$^2$ culture flask; Falcon, Franklin Lakes, NJ). Extra flasks were seeded at the same density so that cell growth could be monitored, and after 4–5 days of culture, cells formed a confluent monolayer and were in transition from exponential growth to the plateau phase. At this time, the cell density was approximately $1.2 \times 10^5$ cells/cm$^2$ and the cells were used for fatty acid and hypothermia treatments.

### Fatty Acid Treatment

The fatty acids used in this study were stearic acid (C18:0), oleic acid (C18:1n-9), linoleic acid (C18:2n-6), α-linolenic acid (C18:3n-3), eicosapentaenoic acid (C20:5n-3; EPA), and docosahexaenoic acid (C22:6n-3; DHA) (all from Sigma Chemical, St. Louis, MO). Stock solutions (100 μM) of the fatty acids were prepared in ethanol and were stored in liquid nitrogen until used. For stearic acid, a 50 mM stock solution was prepared in methanol. Working solutions (10–200 μM) were prepared in DMEM with 1% serum. Cells were washed two times with Hank's balanced salt solution (HBSS, from Gibco) and then treated with the fatty acid or appropriate vehicle solution for 3 h at 37°C in 5% $CO_2$ + 95% air. Following the fatty acid treatment, cells were washed twice with HBSS and once with 1% bovine serum albumin (BSA, from Sigma) before the cold storage solution was added to flasks.

### Effect of Fatty Acid Treatment on Cold-Induced Injury

Confluent cells in 12.5 cm$^2$ flasks were used in these experiments. Cells were treated with 3 ml of 0, 10, 50, 100, or 200 μM solutions of C18:0, C18:1n-9, C18:2n-6, C18:3n-3, C20:5n-3, C22:6n-3, or the appropriate vehicle in DMEM with 1% serum for 3 h at 37°C in 5% $CO_2$ + 95% air. Cells were then washed and flasks received 1.5 ml of DMEM (with 1% serum) and were placed in a circulating water bath at 0°C for 72 or 120 h. The cold-storage medium was not supplemented with additional fatty acids. Flasks were then warmed to 37°C for 3 h because cold-induced injury increases during rewarming. Cell injury was then quantified by measuring the release of the intracellular enzyme LDH into the medium. This method has been used by others to quantify hypothermia-induced injury to endothelial cells and correlates with the loss of cell membrane integrity as measured by the trypan blue assay [Rauen et al., 1993]. Controls consisted of fatty acid or vehicle-treated cells that were washed with HBSS and incubated with 1.5 ml of the cold-storage medium at 37°C for 3 h without the period of cold exposure.

### LDH Assay

After cold-stored cells were rewarmed to 37°C for 3 h, the medium from each flask was collected. Cells were then washed with 3 ml of room temperature HBSS and 2 ml of 1% Triton X-100 in HBSS was added. Cells were incubated for 30 min at 37°C and the lysate was collected and refrigerated until the LDH was measured. The amount of LDH in cell supernatants and cell lysates was determined using a commercially available kit (Roche Diagnostics, Indianapolis, IN). The percentage of total LDH released by cells was calculated by measuring the activity of LDH in the storage medium and dividing by the activity in the medium plus the activity in the cell lysate. In all experiments, corrections were made for LDH activity in DMEM + 1% serum and the lysis buffer.

### Total C18:0 or C22:6n-3 Content of Fatty Acid-Treated Cells

The total C18:0 or C22:6n-3 content of endothelial cells was measured to determined the extent of the fatty acid incorporation during the 3 h treatment that preceded hypothermia. Confluent cells in 75 cm$^2$ culture flasks were treated with 200 μM stearic acid, 0.4% methanol (control), 50 μM DHA, or 0.05% ethanol (control) in DMEM + 1% serum for 3 h at 37°C. Cells from each flask were then rinsed two times with HBSS and once with 1% BSA in HBSS to remove any residual serum fatty acids. Cells were scraped in 15 ml of HBSS, combined from five replicate flasks and collected by centrifugation at 200*g* for 5 min. The lipids were then extracted from the cell pellet according to the method of Folch et al. [1957] and transmethylated using sodium methoxide [Bannon et al., 1982]. Fatty acid methyl esters were analyzed on a Shimadzu GC2010 gas chromatograph (Shimadzu Corporation, Columbia, MD) equipped with an automatic sample injector, flame ionization detector and 0.25 mm × 30 m Stabilwax capillary column (Resteck, Belfont, PA). For optimum fatty acid separation by gas chromatography, the oven temperature program was set at 180°C for 2 min, 180–240°C at 3°C/min and

240–245°C for 1°C/min. Cell fatty acids were identified and quantified against authentic standard fatty acid methyl esters (Nu-Check Prep, Elysian, MN). The unsaturation index of endothelial cell lipids was calculated by the summation of the percentage of each fatty acid in the sample multiplied by the number of double bonds in that fatty acid.

### Changes to Lipid Fractions During Fatty Acid Treatment

Cell lipid fractions were analyzed to determine if the fatty acid treatments significantly changed the size of the free fatty acid, neutral and phospholipid pools before exposing cells to hypothermia. Briefly, confluent cells in 75 $cm^2$ culture flasks were treated with C18:0 or C22:6n-3 as previously described. The pellet from five flasks was combined and lipids were extracted and then fractionated on Silica Gel thin layer chromatography (TLC) plates using a solvent system of petroleum ether:ethyl ether: acetic acid (84:15:1, vol/vol) as described by Karman et al. [1997]. Free fatty acid, neutral lipid and phospholipid fractions were identified by running standards of DHA, cholesterol, and phosphatidylcholine [Ruiz and Ochoa, 1997]. The separated lipid fractions were stained by iodine vapors from sodium iodide, scraped, extracted, esterified, and then analyzed by GLC as described in the preceding section.

### Effect of Fatty Acid and Anti-Oxidant Treatment on Cold-Induced Oxidative Stress

Confluent cells in 75 $cm^2$ culture flasks were treated with fresh growth medium or growth medium supplemented with 200 μM stearic acid, 50 μM DHA, 50 μM DHA + 50 μM vitamin E, or 50 μM DHA + 100 μM of the iron chelator deferoxamine (DFO) for 3 h at 37°C. Cells were washed two times with HBSS and flasks received 4 ml of fresh HBSS and were stored at 0°C for 120 h. Flasks were rewarmed to 37°C for 3 h to maximize the generation of LPO products and cells were scraped and centrifuged at 200*g* for 5 min. LPO products were estimated by measuring the thiobarbituric acid-reactive substances (TBARS).

### TBA Assay

Lipid peroxidation products were estimated in the cell pellet by measuring TBARS. Briefly, samples were resuspended in 0.3 ml of 1% Triton X-100 in water and 0.2 ml aliquots were mixed with 1 ml of 0.8% TBA in 0.5 N HCl in 30% trichloroacetic acid. The reaction mixture was heated to 95°C for 1 h, allowed to cool to room temperature and then was extracted with 1.5 ml of water-saturated *n*-butanol. Samples were centrifuged for 20 min at 2,000*g* and absorbance of the *n*-butanol (upper phase) was measured at 532 nm against a blank consisting of a lysis buffer that had been processed in the same manner as the experimental samples. The results were compared to a standard curve prepared with malonyldialdehyde (MDA) and were expressed as nmoles MDA per mg protein.

### Effect of Anti-Oxidants on DHA-Enhanced Cold Injury

Confluent cells in 12.5 $cm^2$ flasks were used in these experiments. Cells were treated with 3 ml of 0, 10, 50, or 100 μM DHA plus vitamin C (0, 250, or 1,000μM), DFO (0, 10, or 100 μM), vitamin E (0, 10, or 100 μM), or the hydroxyl scavenger butylated hydroxytoluene (BHT-0, 10, or 100 μM) for 3 h at 37°C in 5% $CO_2$ + 95% air. The control cells were treated with the appropriate concentration of vehicle. Cells were then washed 2 times with HBSS and flasks received 1.5 ml of DMEM (with 1% serum) and were placed in a circulating water bath at 0°C for 72 h. The cold-storage medium was not supplemented with additional fatty acids or antioxidants. Flasks were then warmed to 37°C for 3 h as the cold-induced injury increases during rewarming. Cell injury was then quantified by measuring the release of the intracellular enzyme LDH into the medium. Additional controls consisted of fatty acid or vehicle-treated cells that were washed with HBSS and incubated with 1.5 ml of the cold-storage medium at 37°C for 3 h without the period of cold exposure.

### Statistical Methods

All data were plotted as mean values ± stanstandard errors. Statistical analysis was carried out using the InStat program from GraphPad Software (San Diego, CA). Analysis of variance with Tukey's post test was used to compare multiple treatments.

## RESULTS

### Effect of Fatty Acid Treatments on Cold-Induced Cell Injury

Figure 1 shows the effect of pre-hypothermia fatty acid treatments on the percentage of LDH released by damaged cells prior to cold storage

or following their subsequent exposure to 0°C in a fatty acid-free medium for 72 or 120 h and their return to 37°C for 3 h (Fig. 1A, B or C, respectively). LDH released by cells due to the treatments alone ranged from 1.9% to 9.5% (Fig. 1A) and there were no changes in cell morphology. Following 0°C storage, LDH released by cells increased with time at 0°C in all treatment groups (Fig. 1B,C). Only the C18:0 treatments significantly reduced cell injury after 72 or 120 h (50% reduction, $P < 0.01$) when compared to vehicle-treated hypothermic controls. Contrastingly, all other fatty acid treatments, including C18:1n-9, C18:2n-6, C18:3n-3, C20:5n-3 or C22:6n-3, increased the LDH release by cells in a manner that increased with cold exposure time, the fatty acid concentration and the degree of fatty acid unsaturation. Vehicle treatments (methanol-control only is shown) and all 10 μM fatty acid treatments did not change 72 h cold-induced LDH release significantly. Subsequent experiments were performed with stearic acid or DHA since these fatty acids protected cells from the cold or induced maximum sensitivity to hypothermia, respectively.

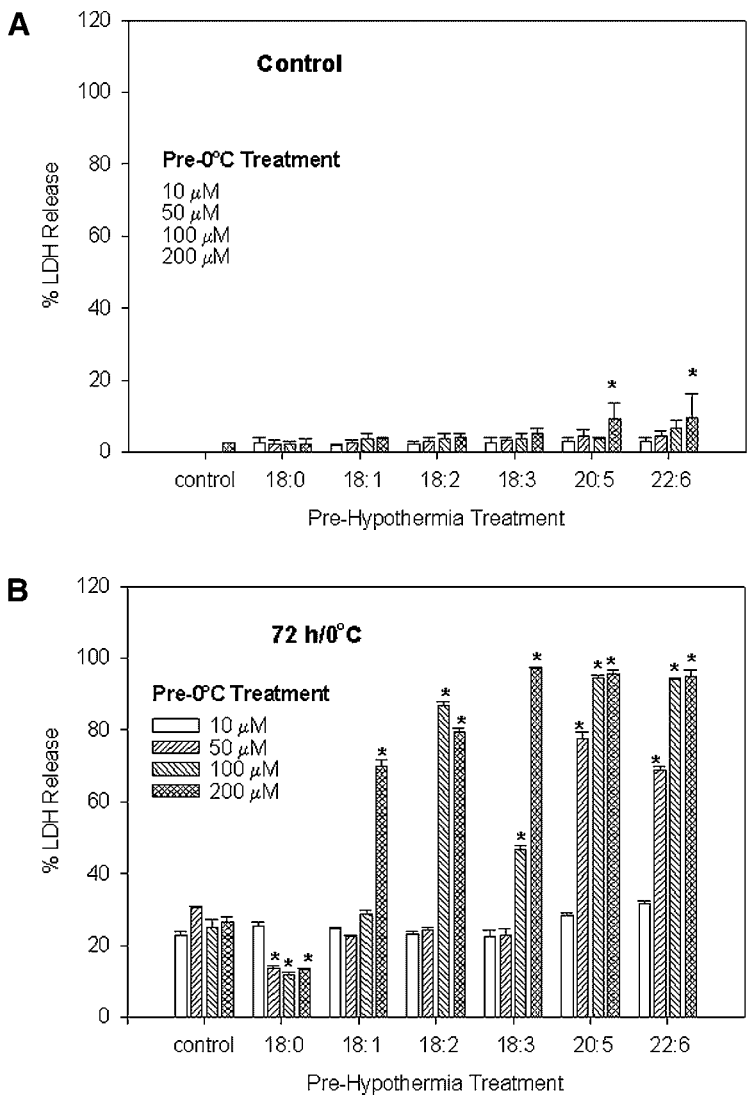


**Fig. 1.** Effect of supplemental fatty acid concentration and time at 0°C on cellular LDH release. **A**: Cells treated with fatty acids without subsequent 0°C storage (controls); (**B**) treated cells stored at 0°C for 72 h; (**C**) treated cells stored at 0°C for 120 h. 18:0 (stearic acid), 18:1n-9 (oleic acid), 18:2n-6 (linoleic acid), 18:3n-3 (linolenic acid), 20:5n-3 (EPA), 22:6n-3 (DHA). *$P < 0.05$ versus vehicle-treated control. Each bar represents mean value ± standard error of at least six samples from three independent experiments.

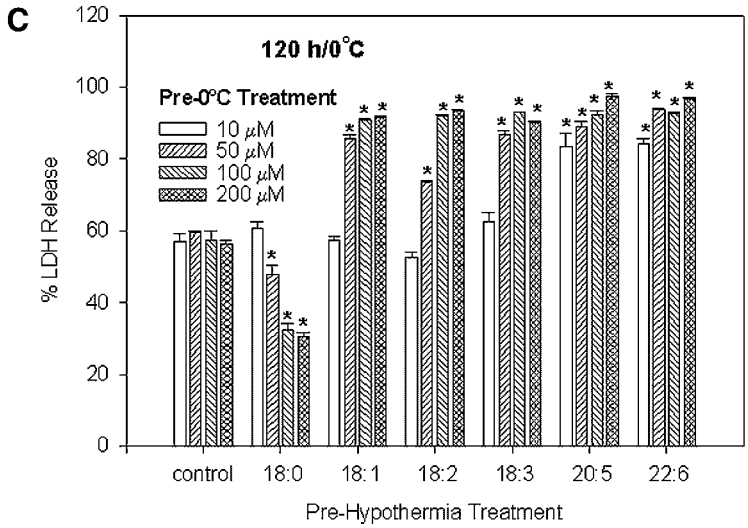


**Fig. 1.** (*Continued*)

### Total C18:0 and C22:6n-3 Content and Changes to Lipid Fractions of Fatty Acid-Treated Cells

Approximately 72% of total cellular fatty acids were found in phospholipids, 8% in neutral lipids, and 5% as free fatty acids. The remaining 15% were unidentified. Vehicle, stearic acid or DHA treatments for 3 h did not change these proportions. The total C18:0 and C22:6n-3 content of untreated, vehicle-treated, stearic acid or DHA-treated endothelial cells was measured to determine the fatty acid uptake by cells (Fig. 2A). The lipids of untreated or vehicle-treated cells (ethanol-control only is shown) were not significantly different and were composed of 19.8% C18:0 and 3.6% C22:6n-3. Treatment of cells with 200 μM stearic acid for 3 h increased C18:0 to 29.6% of the total lipid ($P < 0.01$) and the amount of C22:6n-3 was unchanged (2.4%, $P =$ ns). This was accompanied by a decrease in the unsaturation index of cellular total fatty acids from 121.9 to 92.9. Figure 2B shows that the decrease in unsaturation resulted from an overall increase in saturated fatty acids and a significant decrease in PUFA. Monounsaturated fatty acids were unchanged. Treatment of cells with 50 μM DHA increased C22:6n-3 in cells to 12.1% of the total lipid ($P < 0.01$, Fig. 2A) and the unsaturation index to 166.8 while total C18:0 decreased to 14.9% ($P < 0.01$). The DHA treatment decreased the total saturated fatty acid content

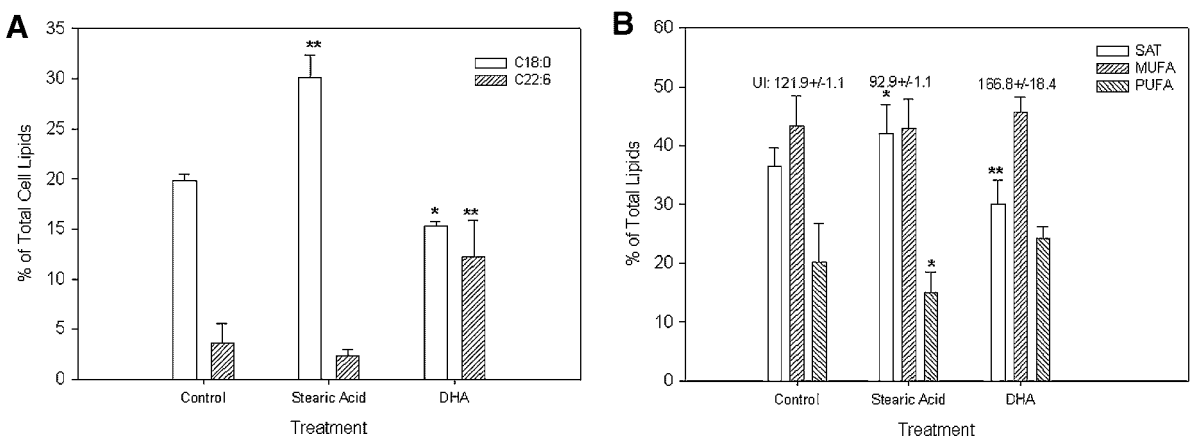


**Fig. 2.** **A**: C18:0 or C22:6n-3 content or (**B**) saturated (SAT), monounsaturated (MUFA), or polyunsaturated fatty acid (PUFA) content of vehicle control, stearic acid or DHA-treated endothelial cells. Unsaturation index (UI). $^{*}P < 0.05$, $^{**}P < 0.01$ versus vehicle-treated control. Each bar represents mean value ± standard error of at least six samples from four independent experiments.

significantly ($P < 0.01$, Fig. 2B), which was offset by minor increases to both mono- and poly-unsaturated fatty acids ($P =$ ns).

### Effect of Fatty Acid and Anti-Oxidant Treatments on Cold-Induced LPO

The effect of fatty acid and anti-oxidant treatments on cold-induced LPO is shown in Table I. Cells treated with growth medium and stored at 0°C for 120 h and rewarmed to 37°C for 3 h showed a threefold increase in TBARS generation ($P < 0.001$) when compared to TBARS generated in untreated non-stored cells. Increasing the total C18:0 content of cells with 200 μM stearic acid reduced TBARS generation when compared to untreated cold-stored cells (52% reduction, $P < 0.05$). Contrastingly, increasing the C22:6 content of cells by treating them with 50 μM DHA at 37°C for 3 h significantly increased TBARS generation during subsequent hypothermia and rewarming (41% increase, $P < 0.01$). The increase in TBARS generation in DHA-cells co-treated with vitamin E or the iron chelator DFO was significantly reduced when compared to cells treated with DHA alone (64% and 54%, respectively, $P < 0.001$).

### Effect of Fatty Acid and Anti-Oxidant Treatments on Cold-Induced Cell Injury

To determine if the observed antioxidant effects of vitamin E or DFO translate into protection from hypothermic injury or a reduction in DHA-induced sensitization to cold, endothelial cells were treated with 0, 25, 50, or 100 μM DHA + 0, 10, or 100 μM vitamin E, DFO or BHT, or 0, 0.25, or 1.0 mM vitamin C. Cells were then stored at 0°C for 72 h and rewarmed to 37°C for 3 h before LDH release was measured. The DHA treatments increased cold-induced LDH release in a concentration-dependent manner and the increase was approximately twofold with 100 μM DHA (Fig. 3A–D). Vitamin E, DFO, or BHT prevented up to 75%, 70%, or 85% ($P < 0.01$), respectively, of the LDH release by untreated cold-stored cells and reduced cold injury in DHA-treated cells by up to 89%, 78%, or 87% ($P < 0.01$), respectively. Contrastingly, vitamin C did not protect untreated cells at 0°C but reduced cold injury to DHA-treated cells by only 37% ($P < 0.01$) (Fig. 3A).

**TABLE I. The Effect of Endothelial Cell Fatty Acid and Antioxidant Treatments on Cold-Induced Oxidative Stress**

| Treatment | TBARS (nmol/mg protein) |
|---|---|
| Control; not stored | 0.90 ± 0.16 |
| Control; 0°C/120 h | 2.77 ± 0.21* |
| 200 μM stearic acid; 0°C/120 h | 1.32 ± 0.22** |
| 50 μM DHA; 0°C/120 h | 3.91 ± 0.29*,** |
| DHA + 50 μM vitamin E; 0°C/120 h | 1.39 ± 0.30**,*** |
| DHA + 100 μM DFO; 0°C/120 h | 1.77 ± 0.28*** |

Values are mean ± standard error of at least 10 samples from 5 independent experiments.
TBARS, thiobarbituric acid-reactive substances; DHA, docosahexaenoic acid; DFO, deferoxamine.
Cells were pre-treated with growth medium (control) or growth medium supplemented with fatty acids or fatty acids + antioxidants for 3 h at 37°C before washing and storage in HBSS at 0°C/120 h.
*$P < 0.001$ versus non-stored controls.
**$P < 0.01$ versus control cells stored at 0°C.
***$P < 0.001$ versus DHA-treated cells stored at 0°C.

## DISCUSSION

This study shows that increasing endothelial cell PUFAs has a detrimental effect on cell survival during hypothermia. The cold-induced release of LDH by fatty acid-treated cells increased with the cold exposure time, the concentration of the supplemental fatty acid and the extent of its unsaturation. The only exception was that stearic acid, which is fully saturated, protected cells from cold injury. In addition, pretreating cells with DHA increased TBARS generation during subsequent 0°C storage and rewarming when compared to alcohol-treated controls that were similarly exposed to cold and then rewarmed. Contrastingly, cells treated with stearic acid before cold storage had significantly less TBARS following cooling and rewarming. These observations are consistent with an oxidative mechanism of injury in cells that increases with the number of fatty acid double bonds available for oxidant reactions. Previous studies have shown that the extent of supplemental fatty acid unsaturation can alter the susceptibility of endothelial cells to peroxidative injury mediated by extracellular $H_2O_2$ [Hart et al., 1991; Vossen et al., 1995] or associated with exposure to 95% $O_2$ [Spitz et al., 1992]. Our study shows that the extent of the supplemental fatty acid unsaturation can modulate oxidative stress and endothelial cell injury induced by hypothermia.

To support these findings, analyses were performed to determine if the endothelial cell fatty acid composition reflected the fatty acid treatments. The C18:0 and C22:6n-3 composition of

our cultured bovine aortic endothelial cells was 19.8% and 3.6%, respectively. This compares with reported values ranging from 12.8% to 17.8% for C18:0 and 1.4% to 3.5% for C22:6n-3 from total lipid, phospholipid or plasma membrane lipids of human or porcine endothelial cells [Block et al., 1989; Cader et al., 1995; Vossen et al., 1995; Cansell et al., 1997; Mazière et al., 1998]. Supplementing the culture medium with stearic acid for 3 h increased the C18:0 content of cells to 29.6% and reduced the unsaturation index by decreasing the amount of cellular PUFA. MUFA were unchanged. Contrastingly, Vossen et al. [1991] showed that the long-term culture of endothelial cells with stearic acid increased the unsaturation index of these cells by increasing the arachidonic acid (C20:4n-6) content in addition to the C18:0. In our experiments, DHA supplementation increased the C22:6n-3 content of the cells and decreased C18:0 from 19.8% to 14.9% of total cellular lipids. The DHA treatment increased the unsaturation index of the cells without significantly changing the MUFA or PUFA content, which suggests that some of the increase in C22:6n-3 was at the expense of other PUFAs that contained fewer double bonds. For example, the arachidonic acid (20:4n-6) content

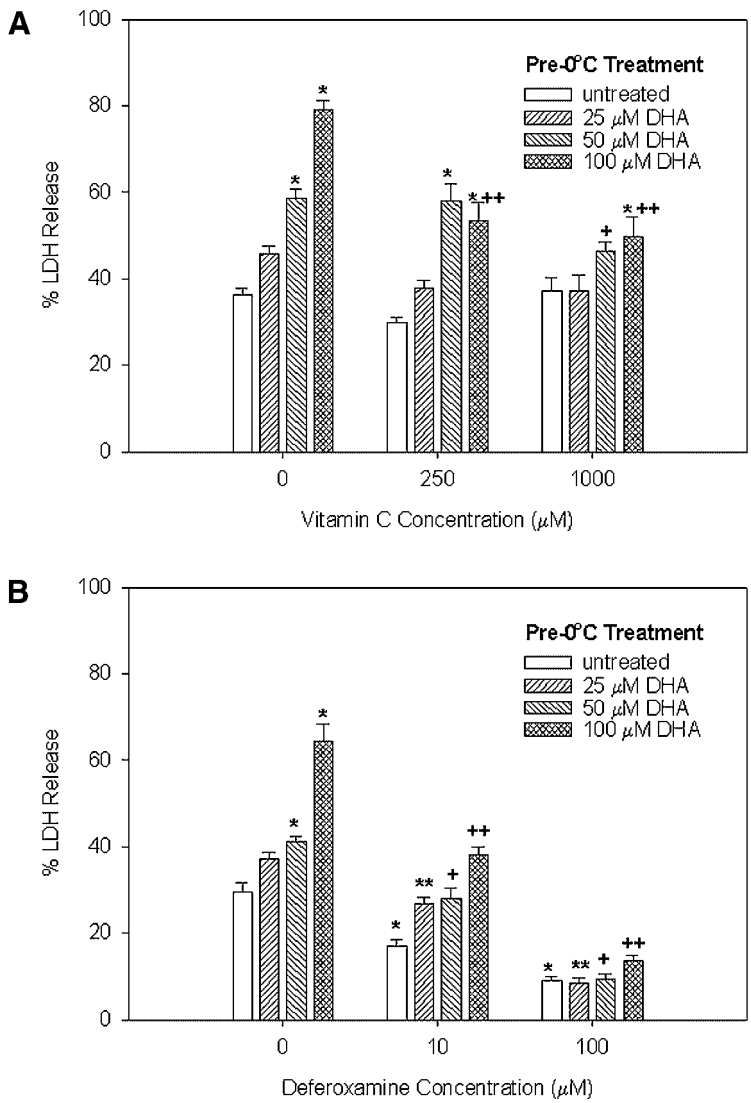


**Fig. 3.** % LDH release of DHA-treated endothelial cells pre-treated with antioxidants (**A**) vitamin C, (**B**) DFO, (**C**) vitamin E, or (**D**) BHT before storage at 0°C for 72 h. $^{*}P < 0.01$ versus untreated control cells stored at 0°C; $^{**}P < 0.01$ versus cells treated with 25 μM DHA only; $^{+}P < 0.01$ versus cells treated with 50 μM DHA only; $^{++}P < 0.01$ versus cells treated with 100 μM DHA only. Each bar represents mean value ± standard error of at least 10 samples from 3 independent experiments.

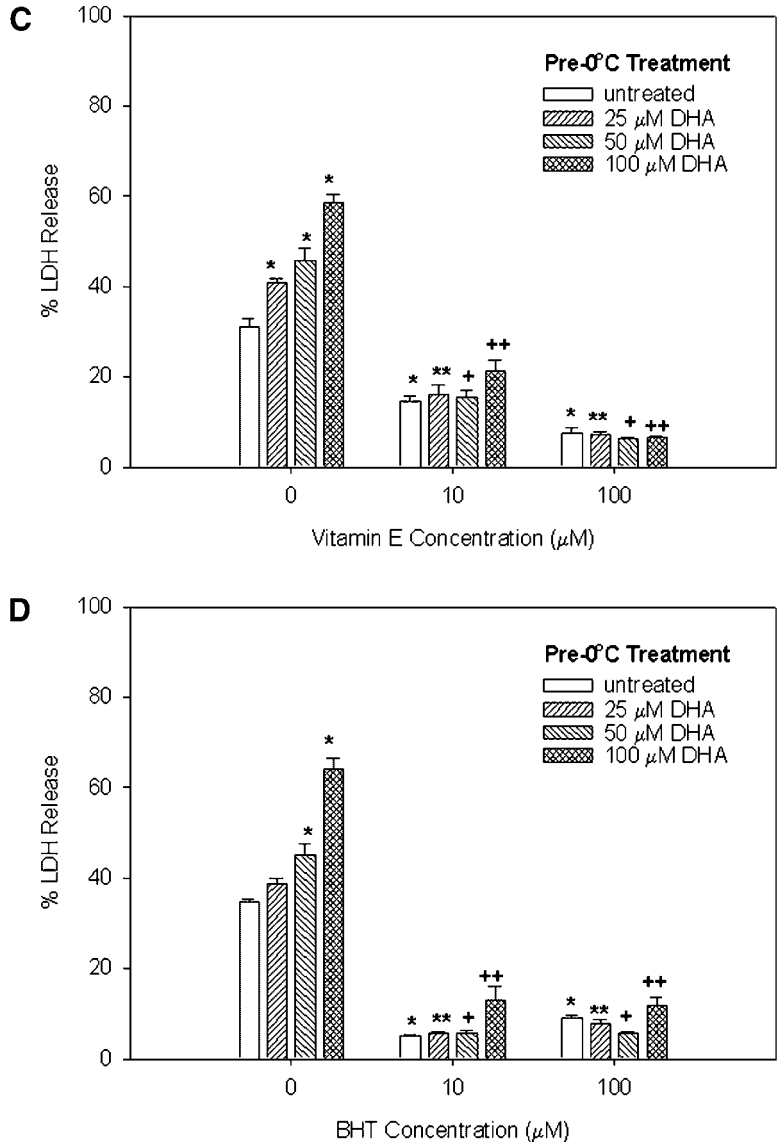


**Fig. 3.** (*Continued*)

of the cells in our study decreased from $7.7 \pm 0.2\%$ to $6.2 \pm 0.2\%$ ($P < 0.01$, data not shown) following the DHA treatment. This is in agreement with observations by others that DHA supplementation of the culture medium increases the C22:6n-3 content of endothelial cells at the expense of cellular arachidonic acid content [Vossen et al., 1991]. It is also the basis for the clinically observed DHA-induced suppression of inflammatory mediators derived from C20:4n-6 [Calder, 2002]. In summary, fatty acid supplementation of the medium induced a corresponding compositional change in the cellular fatty acids. An increase in the unsaturation level of cell fatty acids was accompanied by an increase in cold-induced LPO and cell injury and a decrease in the unsaturation correlated with reductions in cold-induced LPO and injury.

In our studies, increasing the PUFA content failed to improve hypothermic preservation of endothelial cells. Rather, it sensitized cells to cold. The 3-h fatty acid treatment followed by immediate hypothermia may have been insufficient time to incorporate enough PUFAs into membrane phospholipids to exert a beneficial effect on enzyme or transporter function at 0°C. Hart et al. [1991] showed that after treating endothelial cells for 3 h with supplemental oleic acid (C18:1n-9), only 13% of the total cellular

modification was to the phospholipid pool while 72% was to the triglyceride pool. After an additional 24 h without oleic acid, this was largely reversed to 79% and 6%, respectively. In our study, the relative size of the free fatty acid, triglyceride and phospholipid pools did not change during the 3-h treatment, but since modifications to the respective pools likely follow distinct kinetics, we cannot exclude the possibility that the phospholipid pool was insufficiently enriched by the PUFA treatment. PUFA enrichment of the phospholipid pool may not be a requirement for increasing cellular oxidative stress, however. In Hart's study [1991], the extent of the supplemental fatty acid unsaturation was a more important determinant of oxidative injury than the amount of fatty acid incorporated into the phospholipid fraction. In addition, longer exposure of cells to PUFAs increase antioxidant enzymes such as superoxide dismutase, glutathione peroxidase and glutathione *S* transferase [Benito et al., 1997] and increase ferritin-associated iron [Ober and Hart, 1998]. Taken together, the observations suggest that incubating cells with PUFAs may increase their susceptibility to oxidative stress relatively quickly, but the beneficial effects of PUFAs during cold exposure may require more time for their full incorporation into phospholipids or for the up-regulation of antioxidants to neutralize the increased oxidative stress.

The stimulation of oxidative stress by PUFA in vitro is also observed in isolated heart and in vivo models. A 10-min infusion of the isolated rat heart with EPA or DHA emulsions immediately before a period of ischemia and reperfusion reduced cardiac function significantly and increased the release of LDH and TBARS by cells into the perfusion effluent [Schjott et al., 1993]. In mice, the prooxidant effects of fish oil diets high in EPA and DHA included elevated hepatic levels of oxidation products such as TBARS, conjugated dienes, and protein carbonyls [Ibrahim et al., 1997]. Production of TBARS was further stimulated by iron supplements in the fish oil diet but suppressed by the addition of dietary vitamin E [Ibrahim et al., 1997]. Vitamin E is the major lipid-soluble antioxidant of the cell and its function is to prevent or terminate LPO reactions in cell membranes. The in vitro and in vivo observations indicate that vitamin E or other antioxidants may be a necessary adjunct to PUFA-supplementation of cells to minimize the increase in oxidative stress and to facilitate the potentially beneficial effects. This hypothesis is supported by observations of nature, where cold-water fish have a 5 to 6-fold higher vitamin E content than their temperate counterparts to compensate for their increased PUFA content and elevated risk of oxidative stress [Abele and Puntarulo, 2004].

In this study, treating endothelial cells with antioxidants before hypothermia prevented the DHA-induced sensitization to cold. Lipophilic antioxidants prevented up to 85% (Fig. 3) of the LDH release by untreated cells exposed to cold, which indicates that the greatest proportion of hypothermic injury is mediated by oxidative stress rather than one of the other proposed mechanisms, such as cold-induced swelling [Martin et al., 1972; Southard and Belzer, 1980]. This is in agreement with studies by Rauen and de Groot [1998]. Cold-induced oxidative stress may be a result of an increase in production of reactive oxygen species, a temperature-dependent decrease in the activity of antioxidant enzymes or both. The supplementary non-enzymatic antioxidants may be compensating for the reduced antioxidant enzyme activity at 0°C. BHT appeared to be the most potent protective agent, perhaps due to its capacity to increase membrane fluidity and shift the liquid-to-gel transition to lower temperatures [Kruuv et al., 1983] in addition to inhibiting LPO. Supplemental vitamin E was more protective than vitamin C, which is a hydrophilic antioxidant. Vitamin C did not protect control cells at 0°C, which suggests that membrane-associated oxidative stress is the most important contributor to hypothermic injury. However, vitamin C protected cells at high DHA concentrations. This may be due to its role in recycling oxidized vitamin E rather than its role as an antioxidant in aqueous environments. A significant level of LPO and vitamin E depletion may be required before a beneficial effect of vitamin C can be measured. DFO chelates catalytically active iron in the cell, including iron that is loosely bound to membrane phospholipids [Atamna and Ginsburg, 1995]. DFO was protective but slightly less effective than BHT or vitamin E at inhibiting LPO and cell injury, perhaps because its hydrophilic nature prevents it from accessing the interior of cell membranes where LPO

occurs. Also, some cold-induced LPO may be initiated by free iron-independent means, such as through free heme, which is lipid soluble and perhaps less accessible to DFO [Balla et al., 1991].

Several organ preservation studies have shown that antioxidants may be beneficial. DFO was effective in reducing oxidative injury in cold-ischemic [Dobsak et al., 2002; Wickens et al., 1987; Menasché et al., 1990] and cold-perfused rat hearts [Ely et al., 1992; Magni et al., 1993], in cold-ischemic kidneys [Fuller et al., 1986; Green et al., 1986; Healing et al., 1989] and in the cold-perfused rabbit leg model [Iyengar et al., 1990]. Also, vitamin E reduced hypothermia-induced nerve injury in humans [Panjwani et al., 2003] and its addition to organ storage media improved preservation of the cold-ischemic rat lung [Baker et al., 1999], liver [Kumamoto et al., 1999], and kidney [Gower et al., 1987].

In summary, we have shown that a major effect of ultraprofound hypothermia on endothelial cells is oxidative injury that predominantly targets the lipids and damages cell membranes. The level of unsaturation of cell lipids modulates cold-induced oxidative stress and cell injury. Supplementing cells with stearic acid reduces the unsaturation index and is protective during hypothermia. Conversely, supplementing cells with PUFA, especially DHA, increases the unsaturation index and sensitizes cells to the cold. The cold-induced LPO and membrane damage is prevented by treating cells with lipophilic antioxidants and iron chelators. Although no protection was observed in vitro by increasing endothelial cell PUFA content prior to hypothermia, the results suggest that any potential beneficial effects of PUFA would require a simultaneous treatment with antioxidants. Further studies are needed to determine if fatty acid supplemented organ perfusates or cell preservation media can decrease unsaturation levels of cell lipids at hypothermic temperatures and protect against cold-induced oxidative stress.

## REFERENCES


Abele D, Puntarulo S. 2004. Formation of reactive species and induction of antioxidant defence systems in polar and temperature marine invertebrates and fish. Comp Biochem Physiol A 138:405–415.

Allen BS, Veluz JS, Buckberg GD, Aeberhard E, Ignarro LJ. 2003. Deep hypothermic circulatory arrest and global reperfusion injury: Avoidance by making a pump prime reperfusate-a new concept. J Thorac Cardiovasc Surg 125:625–632.

Atamna H, Ginsburg H. 1995. Heme degradation in the presence of E glutathione. A proposed mechanism to account for the high levels of non-heme iron found in the membranes of hemoglobinopathic red blood cells. JBC 270:24876–24883.

Baker CJ, Longoria J, Gade PV, Starnes VA, Barr ML. 1999. Addition of a water-soluble alpha-tocopherol analogue to University of Wisconsin solution improves endothelial viability and decreases lung reperfusion injury. J Surg Res 86:145–149.

Balla G, Vercellotti GM, Muller-Eberhard U, Eaton J, Jacob HS. 1991. Exposure of endothelial cells to free heme potentiates damage mediated by granulocytes and toxic oxygen species. Lab Invest 64:648–655.

Bannon CD, Breen GJ, Craske JD, Hai NT, Harper NL, O'Rourke KL. 1982. Analysis of fatty acid methyl esters with high accuracy and reliability: III. Literature review of and investigations into the development of rapid procedures for the methoxide-catalysed methanolysis of fats and oils. J Chromatogr 247:71–89.

Benito S, Fernandez Y, Mitjavila S, Moussa M, Anglade F, Periquet A. 1997. Phospholipid fatty acid composition affects enzymatic antioxidant defenses in cultured Swiss 3T3 fibroblasts. Redox Rep 3:281–286.

Bernard SA, Buist M. 2003. Induced hypothermia in critical care medicine: A review. Crit Care Med 31:2041–2051.

Block ER, Patel JM, Edwards D. 1989. Mechanism of hypoxic injury to pulmonary artery endothelial cell plasma membranes. Am J Physiol Cell Physiol 257(26): C223–C231.

Cader AA, Butterfield DA, Watkins BA, Chung BH, Hennig B. 1995. Electron spin resonance studies of fatty acid-induced alterations in membrane fluidity in cultured endothelial cells. Int J Biochem Cell Biol 27:665–673.

Calder PC. 2002. Dietary modification of inflammation with lipids. Proc Nutr Soc 61:345–358.

Camara AKS, Riess ML, Kevin LG, Novalija E, Stowe DF. 2004. Hypothermia augments reactive oxygen species detected in the guinea pig isolated perfused heart. Am J Physiol Heart Circ Physiol 286:H1289–H1299.

Cansell M, Gouygou J-P, Jozefonvicz J, Letourneur D. 1997. Lipid composition of cultured endothelial cells in relation to their growth. Lipids 32:39–44.

Chiesa R, Astore D, Piccolo G, Melissano G, Jannello A, Frigerio D, Agrifoglio G, Bonalumi F, Corsi G, Costantini Brancadoro S, Novali C, Locati P, Odero A, Pirrelli S, Cugnasca M, Biglioli P, Sala A, Polvani G, Guarino A, Biasi GM, Mingazzini P, Scalamogna M, Mantero S, Spina G, Prestipino F, Sirchia G. 1998. Fresh and cryopreserved arterial homografts in the treatment of prosthetic graft infections: Experience of the Italian collaborative vascular homograft group. Ann Vasc Surg 12:457–462.

Crowe DM, Hurley JV, Mitchell GM, Niazi Z, Morrison WA. 1998. Long-term studies of cold-stored rabbit femoral artery and vein autografts. Br J Plast Surg 51:291–299.

Dae MW, Gao DW, Sessler DI, Chair K, Stillson CA. 2002. Effect of endovascular cooling on myocardial temperature, infarct size, and cardiac output in human-sized pigs. Am J Physiol Heart Circ Physiol 282:H1584–H1591.

Davignon J, Ganz P. 2004. Role of endothelial dysfunction in atherosclerosis. Circulation 109(Suppl III):III-7–III-32.

Day DJ, Rayburn BK, Gaudin PB, Baldwin III WM, Lowenstein CJ, Kasper EK, Baughman KL, Baumgartner WA, Hutchins GM, Hruban RH. 1995. Cardiac allograft vasculopathy: The central pathogenetic role of ischemia-induced endothelial cell injury. J Heart Lung Transplant 14:S142–S149.

Dobsak P, Siegelova J, Wolf JE, Rochette L, Eicher JC, Vasku J, Kuchtickova S, Horky M. 2002. Prevention of apoptosis by deferoxamine during 4 hours of cold cardioplegia and reperfusion: In vitro study of isolated working rat heart model. Pathophysiology 9:27–32.

Drobnis EZ, Crowe LM, Berger T, Anchordoguy TJ, Overstreet JW, Crowe JH. 1993. Cold shock damage is due to lipid phase transitions in cell membranes: A demonstration using sperm as a model. J Exp Zool 265: 432–437.

Ely D, Dunphy G, Dollwet H, Richter H, Sellke F, Azodi M. 1992. Maintenance of left ventricular function (90%) after twenty-four-hour heart preservation with deferoxamine. J Free Radic Biol Med 12:479–485.

Folch J, Lees M, Sloane-Stanley GH. 1957. A simple method for the isolation and purification of total lipids from animal tissues. J Biol Chem 226:497–509.

Fuller BJ, Myles M, Healing G, Simpkin S, Palmer A, Lunec J, Green CJ. 1986. Investigation of protective agents against oxidative stress during hypothermic storage of pig kidneys. Cryo-Lett 7:102–112.

Gall KL, Smith SE, Willmette CA, O'Brien MF. 1998. Allograft heart valve viability and valve processing variables. Ann Thorac Surg 65:1032–1038.

Gower J, Fuller B, Green C. 1987. Lipid peroxidation in the cortex and medulla of rabbit kidneys subjected to cold ischaemia and the value of protective agents. Free Radic Res Commun 3:107–115.

Green CJ, Healing G, Simpkin S, Fuller BJ, Lunec J. 1986. Reduced susceptibility to lipid peroxidation in cold ischemic rabbit kidneys after addition of desferrioxamine, mannitol, or uric acid to the flush solution. Cryobiology 23:358–365.

Hansen TN. 1993. Correlation between chilling-induced injury in human cells and phospholipid membrane phase transition. Transplant Proc 25:3179–3181.

Hart CM, Tolson JK, Block ER. 1991. Supplemental fatty acids alter lipid peroxidation and oxidant injury in endothelial cells. Am J Physiol Lung Cell Mol Physiol 260(4):L481–L488.

Hayashida N, Ikonomidis JS, Weisel RD, Shirai T, Ivanov J, Carson SM, Mohabeer MK, Tumiati LC, Mickle DAG. 1994. The optimal cardioplegic temperature. Ann Thorac Surg 58:961–971.

Hazel JR. 1984. Effect of temperature on the structure and metabolism of cell membranes in fish. Am J Physiol Regul Integr Comp Physiol 246(15):R460–R470.

Healing G, Green CJ, Simpkin S, Fuller BJ, Lunec J. 1989. Inhibition of lipid peroxidation in renal tissue: In vitro studies with desferrioxamine, mannitol and uric acid. Cryo-Lett 10:7–16.

Hochachka PW. 1986. Defense strategies against hypoxia and hypothermia. Science 231:234–241.

Holzer M. 2002. Mild therapeutic hypothermia to improve the neurologic outcome after cardiac arrest. N Engl J Med 346:549–556.

Holzer M, Bernard SA, Hachimi-Idrissi S, Roine RO, Sterz F, Müllner M. 2005. Hypothermia for neuroprotection after cardiac arrest: Systematic review and individual patient data meta-analysis. Crit Care Med 33:414–418.

Hosenpud JD, Bennett LE, Keck BM, Boucek MM, Novick RJ. 2001. The registry of the international society for heart and lung transplantation: Eighteenth official report-2001. J Heat Lung Transplant 20:805–815.

Huang H, Salahudeen AK. 2002. Cold induces catalytic iron release of cytochrome P-450 origin: A critical step in cold storage-induced renal injury. Am J Transplant 2:631–639.

Ibrahim W, Lee U-S, Yeh C-C, Szabo J, Bruckner G, Chow CK. 1997. Oxidative stress and antioxidant status in mouse liver: Effects of dietary lipid, vitamin E and iron. J Nutr 127:1401–1406.

Iyengar J, George A, Russell JC, Das DK. 1990. The effects of an iron chelator on cellular injury induced by vascular stasis caused by hypothermia. J Vasc Surg 12:545–551.

Karman RJ, Gupta MP, Garcia JGN, Hart CM. 1997. Exogenous fatty acids modulate the functional and cytotoxic responses of cultured pulmonary artery endothelial cells to oxidant stress. J Lab Clin Med 129:548–556.

King RC, Kron IL, Kanithanon RC, Shockey KS, Spotnitz WD, Tribble CG. 1998. Hypothermic circulatory arrest does not increase the risk of ascending thoracic aortic aneurysm resection. Ann Surg 227:702–707.

Kruuv J, Glofcheski D, Cheng K-H, Campbell SD, Al-Qysi HMA, Nolan WT, Lepock JR. 1983. Factors influencing survival and growth of mammalian cells exposed to hypothermia. I. Effects of temperature and membrane lipid perturbers. J Cell Physiol 115:179–185.

Kumamoto Y, Suematsu M, Shimazu M, Kato Y, Sano T, Makino N, Hirano KI, Naito M, Wakabayashi G, Ishimura Y, Kitajima M. 1999. Kupffer cell-independent acute hepatocellular oxidative stress and decreased bile formation in post-cold-ischemic rat liver. Hepatology 30:1454–1463.

Lande MB, Donovan JM, Zeidel ML. 1995. The relationship between membrane fluidity and permeabilities to water, solutes, ammonia, and protons. J Gen Physiol 106:67–84.

Livingstone CJ, Schachter D. 1980. Lipid dynamics and lipid-protein interactions in rat hepatocyte plasma membranes. J Biol Chem 255:10902–10908.

Magni F, Panduri G, Paolocci N. 1994. Hypothermia triggers iron-dependent lipoperoxidative damage in the isolated rat heart. J Free Radic Biol Med 16:465–476.

Martin DR, Scott DF, Downes GL, Belzer FO. 1972. Primary cause of unsuccessful liver and heart preservation: Cold sensitivity of the ATPase system. Ann Surg 175:111–117.

Mazière C, Dantin F, Conte M-A, Degonville J, Ali D, Dubois F, Mazière J-C. 1998. Polyunsaturated fatty acid enrichment enhances endothelial cell-induced low-density-lipoprotein peroxidation. Biochem J 336:57–62.

Menasché P, Grousset C, Mouas C, Piwnica A. 1990. A promising approach for improving the recovery of heart transplants. Prevention of free radical injury through iron chelation by deferoxamine. J Thorac Cardiovasc Surg 100:13–21.

Moriyama Y, Iguro Y, Hisatomi K, Yotsumoto G, Yamamoto H, Toda R. 2001. Thoracic and thoracoabdominal aneurysm repair under deep hypothermia using subclavian arterial perfusion. Ann Thorac Surg 71:29–32.

Ober MD, Hart CM. 1998. Attenuation of oxidant-mediated endothelial cell injury with docosahexaenoic acid: The role of intracellular iron. Prostaglandins Leukot Essent Fatty Acids 59:127–135.

Panjwani U, Yadav DK, Kumar A, Singh SB, Selvamurthy W. 2003. Effect of vitamin C and E supplementation in modulating the peripheral nerve conduction following cold exposure in humans. Int J Biometeorol 48:103–107.

Pukacki F, Jankowski T, Gabriel M, Oszkinis G, Krasinski Z, Zapalski S. 2000. The mechanical properties of fresh and cryopreserved arterial homografts. Eur J Vasc Endovasc Surg 20:21–24.

Quinn PJ. 1985. A lipid-phase separation model of low-temperature damage to biological membranes. Cryobiology 22:128–146.

Rauen U, de Groot H. 1998. Cold-induced release of reactive oxygen species as a decisive mediator of hypothermia injury to cultured liver cells. Free Radic Biol Med 24:1316–1323.

Rauen U, Hanssen M, Lauchart W, Becker HD, DeGroot H. 1993. Energy-dependent injury to cultured sinusoidal endothelial cells of the rat liver in UW solution. Transplantation 55:469–473.

Rauen U, Reuters I, Fuchs A, DeGroot H. 1997. Oxygen free radical-mediated injury to cultured rat hepatocytes during cold incubation in preservation solutions. Hepatology 26:351–357.

Rauen U, Petrat F, Li T, De Groot H. 2000. Hypothermia injury/cold-induced apoptosis—evidence of an increase in chelatable iron causing oxidative injury in spite of low $O_2^-/H_2O_2$ formation. FASEB J 14:1953–1964.

Ruiz JI, Ochoa B. 1997. Quantification in the subnanomolar range of phospholipids and neutral lipids by monodimensional thin-layer chromatography and image analysis. J Lipid Res 38 :1482–1489.

Schjott J, Brekke O-L, Jynge P, Bjerve KS, Hamazak T. 1993. Infusion of EPA and DHA lipid emulsions: Effects on heart lipids and tolerance to ischaemia-reperfusion in the isolated rat heart. Scand J Clin Lab Invest 53:873–882.

Schwab S, Schwarz S, Spranger M, Keller E, Bertram M, Hacke W. 1998. Moderate hypothermia in the treatment of patients with severe middle cerebral artery infarction. Stroke 29:2461–2466.

Seccombe JF, Pearson PJ, Schaff HV. 1994. Oxygen radical-mediated vascular injury selectively inhibits receptor-dependent release of nitric oxide from canine coronary arteries. J Thorac Cardiovasc Surg 107:505–509.

Silvius JR, McElhaney RN. 1980. Membrane lipid physical state and modulation of the $N^+$, $Mg^{2+}$-ATPase activity in *Acholeplasma laidlawii* B. Proc Natl Acad Sci 77:1255–1259.

Southard JH, Belzer FO. 1980. Control of canine kidney cortex slice volume and ion distribution at hypothermia by impermeable anions. Cryobiology 17:540–548.

Spitz DR, Kinter MT, Kehrer JP, Roberts RJ. 1992. The effect of monosaturated and polyunsaturated fatty acids on oxygen toxicity in cultured cells. Pediatr Res 32:366–372.

Thilo L, Träuble H, Overath P. 1977. Mechanistic interpretation of the influence of lipid phase transitions on transport functions. Biochemistry 16:1283–1290.

Thomé C, Schubert GA, Schilling L. 2005. Hypothermia as a neuroprotective strategy in subarachnoid hemorrhage: A pathophysiological review focusing on the acute phase. Neurol Res 27:229–237.

Tsao PS, Aoki N, Lefer DJ, Johnson G, Lefer AM. 1990. Time course of endothelial dysfunction and myocardial injury during myocardial ischemia and reperfusion in the cat. Circulation 82:1402–1412.

Vazquez-Jimenez JF, Qing M, Hermanns B, Klosterhalfen B, Woltje M, Chakupurakal R, Schumacher K, Messmer BJ, von Bernuth G, Seghaye M-C. 2001. Moderate hypothermia during cardiopulmonary bypass reduces myocardial cell damage and myocardial cell death related to cardiac surgery. J Am Coll Cardiol 38:1216–1223.

Von Segesser LK, Marty B, Mueller X, Ruchat P, Gersbach P, Stumpe F, Fischer A. 2001. Active cooling during open repair of thoraco-abdominal aortic aneurysms improves outcome. Eur J Cardiothorac Surg 19:411–416.

Vossen RCRM, van Dam-Mieras MCE, Lemmens PJMR, Hornstra G, Zwaal RFA. 1991. Membrane fatty acid composition and endothelial cell functional properties. Biochim Biophys Acta 1083:243–251.

Vossen RCRM, van Dam-Mieras MCE, Hornstra G, Zwall RFA. 1995. Differential effects of endothelial cell fatty acid modification on the sensitivity of their membrane phospholipids to peroxidation. Prostaglandins Leukot Essent Fatty Acids 52:341–347.

Wickens DG, Li MKW, Atkins G, Fuller BJ, Hobbs KEF, Dormandy TL. 1987. Free radicals in hypothermic rat heart preservation—prevention of damage by mannitol and desferrioxamine. Free Radic Res Commun 4:189–195.

Yacoub M, Rasmi NRH, Sundt TM, Lund O, Boyland E, Radley-Smith R, Khaghani A, Mitchell A. 1995. Surgery for acquired heart disease: Fourteen-year experience with homovital homografts for aortic valve replacement. J Thorac Cardiovasc Surg 110:186–194.

Young JB, Naftel DC, Bourge RC, Kirklin JK, Clemson BS, Porter CB, Rodeheffer RJ, Kenzora JL. 1994. Matching the heart donor and heart transplant recipient. Clues for successful expansion of the donor pool: A multivariable, multiinstitutional report. J Heart Lung Transplant 13: 353–365.

Zieger MAJ, Glofcheski DJ, Lepock JR, Kruuv J. 1990. Factors influencing survival of mammalian cells exposed to hypothermia. IV. Effects of iron chelation. Cryobiology 27:452–464.