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RESPONSES OF HYPERTROPHIED MYOCYTES TO REACTIVE SPECIES: IMPLICATIONS FOR GLYCOLYSIS AND ELECTROPHILE METABOLISM

Brian E. Sansbury^{*,‡}, Daniel W. Riggs^{*}, Robert E. Brainard^{*,‡}, Joshua K. Salabei^{*,†}, Steven P. Jones^{*,‡}, and Bradford G. Hill^{*,†}

^{*}Diabetes and Obesity Center, Institute of Molecular Cardiology, and Department of Medicine, University of Louisville, Louisville, KY

[†]Department of Biochemistry and Molecular Biology, University of Louisville, Louisville, KY

[‡]Department of Physiology and Biophysics, University of Louisville, Louisville, KY

Synopsis

During cardiac remodeling, the heart generates higher levels of reactive species; yet an intermediate “compensatory” stage of hypertrophy is associated with a greater ability to withstand oxidative stress. The mechanisms underlying this protected myocardial phenotype are poorly understood. We examined how a cellular model of hypertrophy deals with electrophilic insults, such as would occur upon ischemia or in the failing heart. For this, we measured energetics in control and phenylephrine (PE)-treated rat neonatal cardiac myocytes (NRCMs) under basal conditions and when stressed with 4-hydroxynonenal (HNE). PE treatment caused hypertrophy as indicated by augmented atrial natriuretic peptide and increased cellular protein content. Hypertrophied myocytes demonstrated a 2.5-fold increase in ATP-linked oxygen consumption and a robust augmentation of oligomycin-stimulated glycolytic flux and lactate production. Hypertrophied myocytes displayed a protected phenotype that was resistant to HNE-induced cell death and a unique bioenergetic response characterized by a delayed and abrogated rate of oxygen consumption and a twofold increase in glycolysis upon HNE exposure. This augmentation of glycolytic flux was not due to increased glucose uptake, suggesting that electrophile stress results in utilization of intracellular glycogen stores to support the increased energy demand. Hypertrophied myocytes also had an increased propensity to oxidize HNE to 4-hydroxynonenic acid and sustained less protein damage due to acute HNE insults. Inhibition of aldehyde dehydrogenase resulted in bioenergetic collapse when myocytes were challenged with HNE. The integration of electrophile metabolism with glycolytic and mitochondrial energy production appears to be important for maintaining myocyte homeostasis under conditions of increased oxidative stress.

Corresponding Author: Bradford G. Hill, Ph.D., 580 South Preston St., Delia Baxter Building II, Rm. 404A, Louisville, KY 40202, Tel: (502) 852-1015, Fax: (502) 852-3663, bradford.hill@louisville.edu.

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Keywords

cardiac hypertrophy; mitochondria; glycolysis; aldehyde dehydrogenase; extracellular flux; 4-hydroxynonenal

Introduction

Electrophilic aldehydes generated from the oxidation of lipids have been detected in nearly all tissues that have experienced oxidative injury. In the heart, they are found in the context of ischemia [1–3], hypertrophy [4–6], and failure [7, 8] and have been classically used as indicators of oxidative stress. The presence of protein adducts with 4-hydroxynonenal (HNE) correlate well with the severity of diastolic dysfunction [9], are localized to myocytes [8], and are consistently elevated and associated with impairment of left ventricular contractile function in heart failure patients [7]. These findings suggest that aldehydes may be active instruments of injury rather than passive footprints of oxidative damage. Nevertheless, how the heart deals with such electrophilic stress is not well understood.

General oxidative stress characterized by increased dihydroethidium staining and the presence of protein-HNE adducts are found relatively early after suprarenal aortic constriction [4]. Similarly, volume overload induced by aortocaval shunting in pigs results in an early increase in aldehydes such as malondialdehyde (MDA) and HNE (peaking at 48 h of overload) [5]. Interestingly, this initial onslaught of reactive species appears to be followed by a transient cardiac phenotype that is relatively protected from redox stress. For example, a model of stable hypertrophy was shown to resist injury caused by ischemia-reperfusion [10, 11] or by perfusion with reactive oxygen species (ROS) [12]; this protection was shown to be due, in part, to heightened antioxidant defenses [10, 11]. The failing heart, however, appears to have lost this adaptation to redox stress, as implicated by a considerable increase in reactive species, oxidative protein damage, and functional decline [7–9, 13].

Remarkable changes in myocardial energetics also occur in the course of cardiac remodeling (reviewed in [14, 15]); however, little is known about how the remodeled myocyte responds to the reactive species generated in the context of ischemia or failure. Understanding this response and how it changes in the course of disease is therefore important because it could lend insight into the mechanisms of myocardial dysfunction and failure. Based on our previous studies, we posit that a major factor underlying aldehyde toxicity stems from their effects on cellular bioenergetics. Isolated cardiomyocytes treated with HNE and subjected to extracellular flux analyses were shown to utilize more oxygen than untreated or nonanal (a non-reactive analog of HNE)-treated cells [16], which matches the pattern of increased oxygen consumption occurring in the post-ischemic [17, 18], hypertrophic, and failing heart [19–21]. This increase in oxygen consumption was due to proton leak as well as ATP-linked processes, suggesting that myocytes respond to aldehydic insults by attempting to increase ATP generation [16]. Thus, it appears that HNE increases cardiomyocyte energy demand and decreases mitochondrial efficiency, which could contribute significantly to cardiomyopathy.

A particularly intriguing hypothesis states that the increased demands of the failing heart lead to a state of energy depletion, through an initial compensatory phase of increased oxygen utilization [22, 23]. With respect to this hypothesis, it is not well understood how mounting oxidative stress or acute insults are handled in the hypertrophied or otherwise “pre-failing” heart. This led us to question how the hypertrophied myocyte handles products generated during oxidative stress, such as HNE. In the present study, we examined energetics in a phenylephrine (PE)-induced cardiomyocyte model of hypertrophy and measured HNE metabolism and the bioenergetic responses of the hypertrophied myocytes to HNE. Our findings suggest that PE-induced cardiomyocyte hypertrophy prevents HNE-induced damage through mechanisms relating to an increased glycolytic reserve and an augmented ability to detoxify lipid-derived aldehydes.

Experimental

Materials

Antimycin A, FCCP (carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone), oligomycin, phenylephrine, mammalian protease inhibitor cocktail and cyanamide were from Sigma (St. Louis, MO, USA) and of the highest grade offered. HNE and ^3H -HNE were prepared on site as previously described [24]. Koningic acid was from TMS Co., Ltd. (Tokyo, Japan). Tritiated 2-deoxyglucose was from Moravsek Biochemicals (Brea, CA). The DC Lowry Protein Assay® was from Biorad (Hercules, CA, USA).

Cardiac myocyte isolation and culture

Neonatal rat cardiac myocytes (NRCM) were isolated from 1- or 2-day-old Sprague-Dawley rats as described [25–28]. The myocytes were then treated with 100 μM phenylephrine (PE) for 48 h to induce hypertrophy [29, 30].

Reverse transcriptase PCR and quantitative real-time PCR

Atrial natriuretic protein (ANP) expression was measured by PCR as described in [31]. Total RNA from isolated cardiomyocytes was extracted with Trizol (Invitrogen, Carlsbad, CA). Total RNA levels were quantified using the ratio of absorbance at 260 nm to 280 nm with the NanoDrop™ 1000 Spectrophotometer (Thermo Scientific). To verify purity of the sample the absorbance ratio from 260 nm to 230 nm (A_{260}/A_{230}) was used. We limited the use of RNA with 260/230 ratio greater than 1.8. Total RNA (1 μg) then was subjected to reverse transcriptase (20 μl final volume) reaction for 30 minutes to synthesize cDNA using IScript™ cDNA synthesis kit (BioRad, Hercules, CA). The sequences used include: ANP sense cctgtgtacagtgcggtgtc; ANP anti-sense aagctgttcagcctagctcc; ribosomal 18s sense aaacggctaccacatccaag; and ribosomal 18s anti-sense cctccaatggatcctcgta. The relative level of mRNA transcripts was quantified by real-time PCR using SYBR® Green (BioRad, Hercules, CA). The data generated was normalized to 18s ribosomal RNA threshold cycle (C_T) values using the C_T comparative method.

Measurement of cellular energetics using the XF24 extracellular flux analyzer

Cellular energetics were measured in intact NRCMs using a Seahorse Bioscience XF24 extracellular flux analyzer (Billerica, MA) as described previously [16]. To allow

comparison between experiments, data are expressed as the rate of oxygen consumption (OCR) in pmol/min and the rate of proton production (PPR) in H^+ /min. For glycolysis assays, we used koniginic acid (KA) to ensure that changes in extracellular acidification were due to glycolysis. For this, three baseline measurements were recorded, followed by the sequential injection of oligomycin (1 $\mu\text{g}/\text{ml}$) and KA (10 $\mu\text{g}/\text{ml}$). Glycolytic flux data were then reported as KA-inhibitable PPR. In some experiments, the data were normalized to the amount of protein present in the well of the microplate. For this, cells were lysed in the 24-well XF plates using 20 $\mu\text{l}/\text{well}$ of lysis buffer containing 20 mM HEPES, pH 7.0, 1 mM EDTA, 1% NP-40, 0.1% SDS and protease inhibitor cocktail followed by protein assay.

Radiolabeled Glucose Uptake

Glucose uptake in intact NRCMs was determined using ^3H -2-deoxy-D-glucose (^3H -DOG). After 48 h of incubation in the absence or presence of PE, the medium was removed and replaced with fresh, serum-free DMEM (5.5 mM glucose) containing ^3H -DOG (2 $\mu\text{Ci}/\text{ml}$) and vehicle (ethanol), HNE (20 μM), or oligomycin (1 $\mu\text{g}/\text{ml}$). The NRCMs were then incubated at 37°C for 3 h. Following incubation, the cells were washed with ice-cold PBS six times to remove adherent radioactivity. The cells were then lysed with lysis buffer containing 20 mM HEPES, pH 7.0, 1 mM EDTA, 1% NP-40, 0.1% SDS, and centrifuged at $13,000\times g$ for 10 min. The supernatant was collected and a small amount was used to determine the protein concentration by the Lowry DC method. The radioactivity was then measured by scintillation counting and normalized to protein.

Lactate Assay

Following 48 h of culture in the absence or presence of PE, the medium was removed and replaced with fresh, serum-free DMEM (5.5 mM glucose) containing vehicle (ethanol), HNE (20 μM), or oligomycin (1 $\mu\text{g}/\text{ml}$). The NRCMs were then incubated at 37°C for 3 h. Following incubation, 50 μl of medium was used to measure lactate levels using a Lactate Assay Kit (Eton Bioscience Inc., San Diego, CA).

Metabolism of HNE in NRCMs

Three to five days after isolation, NRCMs were exposed for 1 h to ^3H -HNE (15 μM HNE) in modified Kreb's Hensleit (KH) buffer, pH 7.4, containing in mM: 10 HEPES, 111 NaCl, 4.7 KCl, 2.0 MgSO_4 , 1.2 Na_2HPO_4 , 1.2 CaCl_2 , 5.5 D-glucose, 1.0 pyruvate. The incubation medium was then filtered through a 0.2 μm filter and unmetabolized HNE and metabolic products of HNE in the incubation medium were resolved by HPLC and quantified by scintillation counting essentially as described in [3, 24]. To determine protein-bound HNE, the cells were scraped in 6% perchloric acid and the protein pellets were obtained by centrifugation at $13,000\times g$ for 10 min. The pellets were then washed with acetone until no radioactivity was detected in the wash. Finally, the protein was resuspended in 100 μl 0.5 M Tris, pH 8.8, containing 1% SDS, the protein concentration was measured by the Lowry DC method, and the radioactivity in the protein sample was measured by scintillation counting.

Statistical analyses

Data are reported as means $\pm$ SEM. Comparisons between two groups were performed with Student's *t*-test. Multigroup comparisons were performed using one-way ANOVA, with Bonferroni correction. The null hypothesis was rejected when $p < 0.05$.

Results

Effects of cardiomyocyte hypertrophy on cellular energetics

Extracellular flux analyses were used to determine the effects of phenylephrine (PE)-induced hypertrophy on cardiomyocyte energetics. NRCMs were treated for 48 h with phenylephrine to induce hypertrophy. As shown in Fig. 1A and B, PE increased transcription of a marker of hypertrophy, atrial natriuretic peptide (ANP), and increased total cellular protein content. After PE exposure, the cells were washed with DMEM and the energetic assays were performed as described in [16]. As shown in Fig. 2A and B, cells treated with PE consumed significantly more oxygen than controls cells at baseline. Addition of oligomycin was used to determine whether this was due to ATP-linked or non-ATP-linked changes in oxygen consumption. As shown in Fig. 2C, the increase in oxygen consumption was due to a 2.5-fold increase in ATP-linked oxygen demand. Non-ATP-linked oxygen consumption, which is likely due to proton leak [16, 32], was not significantly changed by PE treatment (Fig. 2D). While maximal oxygen consumption rates were not changed with PE (Figs. 2B and 2E), the reserve capacity was decreased (Fig. 2F), which appears to be due to the increase in ATP-linked oxygen demand.

To measure changes in glycolysis, the proton production rate (PPR) was used as a surrogate marker of glycolytic flux [33]. To aid in confirming that the changes in PPR were representative of glycolysis, we incorporated the use of koniginic acid (KA) in our glycolysis assays. Briefly, KA is a sesquiterpene antibiotic that forms a covalent adduct with Cys-149 of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), thereby inhibiting the enzyme and glycolysis [34–36]. For these assays, three baseline PPR measurements were recorded, followed by addition of oligomycin. After one measurement, koniginic acid was then injected to inhibit glycolysis, and one more PPR was recorded (Fig. 3A). KA-inhibitable PPR was then used to measure changes in glycolytic flux. Although hypertrophied myocytes showed no increase in basal, KA-inhibitable glycolysis (Fig. 3B), PPR was increased more than twofold when mitochondrial ATP production was inhibited with oligomycin (Figs. 3C).

Cell death induced by electrophile stress

In previous studies, we examined cell death in NRCMs exposed to HNE and found that incubation of cells with 5 μ M HNE for 16 h resulted in ~50% cell death [16]. Using identical conditions, HNE-induced cell death was examined in control and hypertrophied myocytes. As shown in Fig. 4, 16 h of HNE treatment resulted in ~60% loss in cell viability in control myocytes; however, PE-treated myocytes showed a marked protection against cell death caused by HNE.

Energetic responses of hypertrophied myocytes to HNE

Because hypertrophied myocytes had a significantly different energetic profile compared to normal myocytes (Figs. 2 and 3), we next questioned how energetics would change when these myocytes are challenged with HNE. After three baseline measurements, control and PE-hypertrophied myocytes were treated with HNE and the rates of oxygen consumption and proton production (PPR) were measured in time. As shown in Fig. 5A and C, HNE led to a transient, robust increase in OCR and PPR which was followed by rates that fell below baseline. Interestingly, the response of NRCMs that were treated with PE for 48 h differed both temporally and in a manner of extent. The bioenergetic rates in PE-treated myocytes exposed to HNE peaked 40–50 min after that of non-hypertrophied cells (Fig. 5A and C). Shown in Fig. 5B and D are OCR and ECAR traces normalized to baseline energetic rates. Interestingly, the extent to which HNE increased oxygen consumption was largely diminished, but the increase in glycolysis appeared to be conserved. To quantify the overall changes in respiratory activity and glycolysis in control and PE-treated myocytes, the area under the curve (AUC) from baseline was determined. As shown in Fig. 5E and F, PE-treated myocytes used almost twofold less oxygen in the response to HNE and increased their overall rate of glycolytic flux after HNE exposure, as indicated by a 2.3-fold increase in proton production. Pretreatment of control and hypertrophied NRCMs with konigic acid prior to HNE exposure inhibited HNE-induced changes in oxygen consumption and glycolysis (Supplementary Fig. 1).

Effects of HNE on glycolysis and glucose uptake in control and hypertrophied myocytes

To further determine whether the apparent increase in glycolysis as measured by PPR in Fig. 5B, D, and F was due to increased glycolysis, we measured lactate production in cells treated for 3 h with HNE. As shown in Fig. 6A, HNE treatment led to a 1.7-fold increase in lactate production in control cells. Addition of oligomycin to control cells for the 3 h period resulted in a nearly threefold increase in lactate production; this finding is in agreement with the increase in PPR upon oligomycin treatment shown in Fig. 3. Hypertrophied myocytes showed only a modest increase in lactate production compared with controls cells; however, lactate production was increased by 2.4-fold in hypertrophied cells treated with HNE, affirming that hypertrophied myocytes, as compared with control myocytes, utilize glycolysis to a greater extent when challenged with HNE. Oligomycin treatment of hypertrophied cells resulted in a 4.4-fold increase in lactate production over control (non-PE-treated) myocytes, and a 1.5-fold increase in lactate production over oligomycin-treated, non-hypertrophied cells.

To determine if HNE and oligomycin increase glucose uptake by NRCMs, we measured ^3H -DOG uptake in non-hypertrophied and hypertrophied myocytes treated without or with HNE or oligomycin. Interestingly, HNE treatment did not affect ^3H -DOG uptake in either control or PE-treated NRCMs (Fig. 6B); however, oligomycin treatment in both non-PE and PE-treated cells increased ^3H -DOG uptake by more than fourfold.

Metabolism of HNE in hypertrophied NRCMs

Next, we examined HNE metabolism in control and hypertrophied myocytes. As shown in Table 1 and Fig. 7A, the largest route for HNE metabolism in control and PE-treated

myocytes was via glutathione conjugation, followed by oxidation to HNE and reduction to DHN. The most striking difference between control and PE-treated myocytes was the formation of HNA, such that the HNA to HNE ratios were significantly different between the groups (Fig. 7B). Interestingly, this difference in metabolism was associated with a reduction in the amount of protein-bound HNE in the hypertrophied myocytes (Fig. 7C), suggesting that PE-hypertrophied myocytes incur less aldehyde-induced protein damage, which may be attributed to greater dehydrogenase-mediated HNE oxidation.

To test whether loss of aldehyde dehydrogenase activity would affect the bioenergetic response to HNE, we performed experiments using HNE-treated NRCMs in the absence or presence of cyanamide, an inhibitor of aldehyde dehydrogenase. Three baseline oxygen consumption and extracellular acidification rates were recorded, followed by addition of vehicle or cyanamide to the cells. Two more rates were recorded, and then vehicle or HNE was added to the cells for the indicated time. As shown in Supplementary Fig. 2A, addition of cyanamide alone had a modest effect on OCR, but a much greater effect was observed after HNE addition. As previously shown [16], HNE increased the rate of oxygen consumption by more than twofold. Pretreatment of the cells with cyanamide, however, hastened the increase in oxygen consumption and promoted a sudden bioenergetic collapse. Analogous results are shown in the glycolytic flux assays shown in Supplementary Fig. 2B. Interestingly, cyanamide alone increased glycolytic flux by approximately threefold. An even higher transient increase in extracellular acidification rate is shown in the cyanamide +HNE trace; this was followed by an abrupt loss of all energetic activity.

Discussion

Cardiac hypertrophy is an important compensatory response to physiologic and pathological stimuli. A variety of hormonal and hemodynamic stimuli drive the hypertrophic program, leading to increases in myofibrillar content (rather than proliferation) and changes in metabolic phenotype. In particular, the hypertrophy of myocytes occurs concomitantly with an increase in glucose uptake and glycolysis and a later decrease in overall fatty acid metabolism [14, 37]. This switch mimics the metabolic profile observed in the fetal heart [38]. However, little is known about how metabolism changes in the hypertrophied heart under conditions of increased stress caused by reactive species such as oxidants and electrophiles. As a first step to addressing this, we examined energetics and HNE metabolism in control and PE-hypertrophied myocytes. The major findings of this study are: (1) hypertrophied myocytes utilize more oxygen than control myocytes; (2) hypertrophy increases the ability of the myocyte to utilize glycolysis during stress; (3) hypertrophied myocytes are better able to survive electrophilic insults; and (4) hypertrophied myocytes increase their apparent aldehyde dehydrogenase activity and concomitantly demonstrate lower levels of protein-HNE adducts.

In our study, we utilized a well-studied PE model of cardiomyocyte hypertrophy [29, 30]. Phenylephrine is an α_1 -adrenergic agonist that can be used to regulate cardiac metabolism and function. α -adrenergic stimulation produces both inotropic [39] and chronotropic [40] myocardial effects associated acutely with an increase in Ca^{2+} flux [41], phosphatidylinositol turnover [41], and inhibition of cAMP phosphodiesterase activity [42]. These effects are

followed by an increase in the synthetic rates of contractile proteins, brain natriuretic peptide (BNP) and atrial natriuretic protein (ANP) expression, and increases in cellular volume and protein content [29, 30, 43, 44]. Interestingly, PE has been shown to precondition the heart [45–47] and protect mitochondria from calcium overload [48]. In our study, we used ANP message and protein content as positive indicators of the PE-induced hypertrophic response prior to examining energetics and HNE metabolism (Fig. 1A and B).

Using sequential addition of oligomycin, FCCP, and antimycin A in extracellular flux assays, we were able to examine energetics in hypertrophied myocytes and quantify several distinct metabolic parameters (see [16] for further information on extracellular flux assays in primary cardiomyocytes). Inhibition of mitochondrial ATP synthesis with oligomycin was used to determine the extent of ATP-linked and non-ATP-linked utilization, the uncoupling agent FCCP was used to determine the maximal respiratory and reserve capacities, and antimycin A was used to inhibit all electron flux and measure non-mitochondrial oxygen consumption. Interestingly, we found that the increase in basal oxygen consumption in PE-hypertrophied myocytes was due to an increase in ATP demand, with no contribution of proton leak (Fig. 2). While it has been shown that PE increases myocardial oxygen utilization in the nonfailing heart [49], whether this leads to efficient energy production is unknown. Our finding suggests that PE-treated myocytes utilize oxygen in an efficient manner during the hypertrophic response. There was no increase in maximal respiratory capacity in hypertrophied myocytes and a net decrease in the mitochondrial reserve capacity. Consistent with previous studies [50], PE-mediated hypertrophy augmented the rate of glycolysis when oxidative phosphorylation was inhibited (Fig. 3). This suggests that the hypertrophied myocyte is better able to call upon glycolysis when mitochondrial ATP synthesis is lost. Collectively, these data suggest that PE-induced hypertrophy decreases the mitochondrial reserve capacity (due to an increase in basal oxygen consumption) while augmenting the apparent glycolytic reserve.

There may be several reasons for this increase in glycolytic capacity. It has been shown that stimulation of α -adrenergic receptors increases the activity of phosphofructokinase (PFK) in perfused rat hearts [50, 51], suggesting that this rate-limiting enzyme could have been affected in hypertrophied myocytes. Furthermore, in the pressure-overloaded heart, the levels of PFK activators are elevated tenfold, leading to an increase in glycolytic flux [52], and the activity and/or expression of several glycolytic enzymes have been shown to be increased in the hypertrophied heart [14, 53, 54]. Interestingly, HNE exposure increased glycolytic flux to a greater extent in hypertrophied myocytes compared with non-hypertrophied myocytes. Although lactate production mirrored the HNE-induced changes in glycolysis measured by extracellular flux analyses, ^3H -deoxyglucose (^3H -DOG) uptake was unchanged by HNE exposure, indicating that the glucose oxidized by the cell under electrophile stress was derived from intracellular stores—likely glycogen. That glucose derived from glycogen is preferentially oxidized in the heart compared with exogenous glucose [55, 56] therefore suggests that glycogen may be the depot that is first utilized by the heart under conditions of electrophile stress. Although glycogen metabolism in the hypertrophied heart does not differ from the non-hypertrophied heart under non-ischemic conditions [55], glycogen turnover is accelerated in hypertrophied hearts exposed to severe ischemia [57]—a stress where a marked increase in electrophiles such as HNE is observed

[2, 3]. Oligomycin, however, significantly increased ^3H -DOG uptake, indicating that overt loss of mitochondrial ATP synthesis does result in an increased capacity of the cell to import extracellular glucose. Taken together, these findings imply that HNE could promote glycogen turnover, which is then used to provide fuel for the myocyte to survive under conditions of electrophilic stress.

In this study, we also observed that the hypertrophic myocyte responds differently to HNE, i.e., it uses less oxygen after being exposed to HNE than a non-hypertrophied myocyte and appears to rely more on glycolysis during the electrophilic encounter (Fig. 5). This may be due to the fact that, in the hypertrophied heart, glucose oxidation is not in correspondence with glycolytic rates and that mitochondrial glucose oxidation may actually be lower than that found in the non-hypertrophied heart (reviewed in [14]). Hence, it appears that under conditions of electrophile stress, the hypertrophied myocyte utilizes anaerobic glycolytic pathways to a greater extent than the non-hypertrophied myocyte. The bioenergetic response to HNE, in general, was also delayed temporally with respect to that of a normal myocyte. This latency could be explained by differences in how the cell metabolizes electrophiles such as HNE. HNE has been shown to be detoxified by several pathways including oxidation to 4-hydroxynonenic acid (HNA), reduction to dihydroxynonene (DHN), and conjugation to glutathione [24]. In the rat heart, it was shown that the predominant route of metabolism for HNE is via NAD^+ -dependent oxidation to HNA and that mitochondria are centers for such HNE metabolism [3]. HNE metabolism in control and hypertrophied myocytes appeared similar (Table 1) with the exception of differences in the quantities of HNA and HNE, leading to an increased HNA/HNE ratio and decreased protein-HNE adducts in hypertrophied myocytes (Fig. 7). It is intriguing to speculate that the bioenergetic phenomena observed in the PE-hypertrophied myocytes exposed to HNE may be due to better metabolism of toxic aldehydes, leading to preservation of myocardial energetics. Aldehyde dehydrogenase 2, a mitochondrial localized ALDH isoform, has been shown to attenuate myocardial ischemia-reperfusion injury [58, 59] and plays an important role in detoxifying aldehydes in the heart [3, 24]. In our study, ALDH2 expression was not increased over control levels in the hypertrophied myocytes (data not shown), indicating that other factors relating to the activity of the enzyme, such as phosphorylation or other post-translational protein modifications, may be responsible for the observed effects. Indeed, protein kinase C epsilon ($\text{PKC}\epsilon$) has been shown to phosphorylate and activate ALDH2 [60], and phenylephrine and other adrenergic agonists activate $\text{PKC}\epsilon$ and promote their translocation to membrane fractions [61–63].

We propose that the increase in apparent aldehyde dehydrogenase activity may be responsible for the attenuation of protein damage by HNE and could, in part, underlie the energetic changes and protection against cell death observed in PE-treated myocytes after HNE exposure (Fig. 4 and 5). Our finding that ALDH activity is important for maintaining energetic integrity in myocytes exposed to electrophile stress (Supplementary Fig. 2) further supports this concept. An additional factor unveiled by these studies is the glycolytic reserve, which was increased in the hypertrophied myocyte. Although the mitochondrial response to HNE appeared to be diminished, the enhanced ability of PE-treated myocytes to utilize glycolysis may be important during conditions of increased oxidative stress. Taken as a whole, we conclude that HNE metabolism and the ability to sustain glycolysis may be

linked and integral conduits used by myocytes to maintain energetic poise in the face of electrophilic insults. Understanding how each of these factors change during the course of myocardial remodeling should yield further insights into the mechanisms of heart failure.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

ALDH	aldehyde dehydrogenase
ANP	atrial natriuretic peptide
BNP	brain natriuretic peptide
DHN	dihydroxynonene
DMEM	Dulbecco's modified Eagle's medium
ECAR	extracellular acidification rate
FCCP	carbonyl cyanide <i>p</i> -trifluoromethoxyphenylhydrazone
HNA	4-hydroxynonenoic acid
HNE	4-hydroxynonenal
MDA	malondialdehyde
NRCM	neonatal rat cardiomyocyte
OCR	oxygen consumption rate
PE	phenylephrine
PKC	protein kinase C
PPR	proton production rate
GS-X	glutathione conjugates of HNE, including GS-HNE and GS-DHN
XF	extracellular flux

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Figure 1. Markers of myocyte hypertrophy in control and phenylephrine-treated myocytes
Isolated cardiomyocytes were exposed to phenylephrine (PE, 100 μ M) for 48 h, and markers of hypertrophy were measured. (A) Atrial natriuretic peptide (ANP) message as detected by pPCR. (B) Total cellular protein normalized to control cells. $n = 3$ pergroup, $*p < 0.05$.

Figure 2. Hypertrophied myocytes demonstrate an increase in ATP-linked oxygen consumption
 Extracellular flux analysis of NRCMs: **(A)** Oxygen consumption rates (OCR) in control and PE-treated cells. After three baseline measurements, oligomycin (Oligo; 1 μg/ml), FCCP (1 μM), and antimycin A (AA; 10 μM) were injected to assess mitochondrial efficiency, maximal respiratory rate, and non-mitochondrial oxygen consumption. Panel **B** shows data normalized to total protein. **(C)** Measurements of ATP-linked oxygen consumption rate (OCR), **(D)** proton leak, **(E)** maximal OCR, and **(F)** mitochondrial reserve capacity. n = 5 per group, *p<0.05.

Figure 3. Myocyte hypertrophy results in an increased ability to augment glycolysis

Extracellular flux analysis of NRCMs: (A) Proton production rates (PPR) of control and PE-treated cells. After three baseline measurements, oligomycin (Oligo; 1 $\mu\text{g}/\text{ml}$) and koningic acid (KA; 10 $\mu\text{g}/\text{ml}$) were injected to assess KA-inhibitable basal (panel B) and oligomycin-stimulated (panel C) glycolytic flux. $n = 5$ per group, $*p < 0.05$

Figure 4. Phenylephrine-hypertrophied myocytes are protected from HNE-induced cell death
NRCMs were treated without or with PE (100 μ M) for 48 h followed by exposure to HNE (5 μ M) for 16 h. Cell viability was then measured by MTT assay. n = 5 per group, *p<0.05.

Figure 5. Energetic responses of normal and hypertrophied myocytes to HNE

Extracellular flux analyses of NRCMs treated with HNE. After three baseline measurements, the cells were exposed to HNE (20 μ M) for the time shown. (A and B) Oxygen consumption rates (OCR) of NRCMs: panel A, raw OCR data; panel B, data shown as percent of baseline. (C and D) Proton production rates (PPR) indicative of glycolysis in NRCMs: panel C, raw PPR data; panel D, PPR shown as percent of baseline values. (E and F) Area under the curve (AUC) analyses of oxygen consumption (panel E) and proton production (panel F) were used to determine the amount of oxygen consumed and protons produced with each treatment. n = 5 per group, *p<0.05.

Figure 6. Lactate production and 2-deoxyglucose uptake in NRCMs

NRCMs were treated without (white bars) or with PE (100 μ M; black bars) for 48 h followed by incubation in DMEM (5.5 mM D-glucose) in the absence or presence of HNE (20 μ M; +HNE) or oligomycin (1 μ g/ml; +Oligo). (A) Lactate concentration in the medium after 3 h of incubation. (B) 3 H-2-deoxyglucose (3 H-DOG) uptake in NRCMs: NRCMs were treated identically to cells in panel A, and 3 H-DOG (0.2 μ Ci/ml) was included in the medium. Radioactivity in the cells was then measured and normalized to total protein. n = 3–4 per group; P<0.0001 (one-way ANOVA); *p<0.05 vs. CTRL (white bar); #p<0.05 vs. +HNE (white bar); @p<0.05 vs. +Oligo (white bar).

Figure 7. HNE metabolism in control and hypertrophied cardiomyocytes

Isolated NRCMs were exposed to ^3H -HNE (15 μM ; $\sim 70,000$ CPM/well) for 1 h and the metabolites in the medium were resolved by HPLC and quantified by scintillation counting.

(A) Representative radioplots of metabolites extruded by NRCMs treated with radiolabeled HNE: panel **i**, control NRCMs and panel **ii**, phenylephrine (PE; 100 μM)-treated myocytes.

(B) Ratio of HNA to HNE from group data. $N = 4/\text{group}$, $*p < 0.05$. (C) Protein-bound ^3H -HNE quantified from protein isolated from ^3H -HNE myocytes. $n = 4$ per group, $*p < 0.05$.

Table 1HNE metabolism in control and phenylephrine-treated myocytes¹.

Metabolite ²	Control myocytes (% metabolism)	Hypertrophied myocytes (% metabolism)
GS-X	30.0±1.1	31.1±1.0
DHN	6.7±0.5	7.7±0.5
HNA	17.5±1.4	21.0±1.4
HNE	24.9±2.4	20.9±1.9
Other	20.9±0.5	19.3±0.9

¹ NRCMs were treated for 48 h in the absence or presence of 100 μ M phenylephrine to induce hypertrophy. The cells were then exposed to 15 μ M ³H-HNE for 1 h at 37°C. Metabolites in the media were then resolved by HPLC and quantified by scintillation counting. n = 4 (separate isolations per group) $\pm$ SEM.

² GS-X represents the glutathione conjugates of HNE (including GS-HNE and GS-DHN); DHN represents dihydroxynonene, HNA represent 4-hydroxynonenic acid, and HNE is unmetabolized 4-hydroxynonenal.