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High-performance liquid chromatography (HPLC) determination of inosine, a potential biomarker for initial cardiac ischaemia, using isolated mouse hearts

D. FARTHING[1,2], L. XI[3], L. GEHR[4], D. SICA[2], T. LARUS[2], & H. T. KARNES[1]

[1]*Department of Pharmaceutics,* [2]*Department of Clinical Pharmacology and Hypertension,* [3]*Department of Cardiology and* [4]*Department of Pediatrics, VCU Medical Center, Virginia Commonwealth University, Richmond, VA, USA*

Abstract
Each year in the USA approximately 7–8 million patients with non-traumatic chest pain come to hospital emergency rooms. It is estimated that approximately 2–5% of these patients are experiencing cardiac ischaemia, but due to the shortcomings of the available testing methods they are incorrectly diagnosed and discharged without appropriate therapy having been provided. Preliminary data with a globally ischaemic mouse heart model has demonstrated that endogenous inosine might be a potential biomarker of initial cardiac ischaemia before cardiac tissue necrosis. A high-performance liquid chromatographic diode array detection (HPLC-DAD) method was utilized for the detection and quantification of inosine in Krebs–Henseleit (Krebs) buffer solution perfusing from surgically removed and isolated mouse hearts undergoing global cardiac ischaemia. A C_{18} column at a flow rate of 0.6 ml min^{-1} with an aqueous mobile phase of trifluoroacetic acid (0.05% trifluoroacetic acid in deionized water, pH 2.2, v/v) and methanol gradient was used for component separation. The assay detection limit for inosine in Krebs buffer solution was 500 ng ml^{-1} using a 100-µl neat injection. The HPLC results were used to determine total cardiac effluxed inosine into the Krebs effluent for each mouse during oxidative stress and compared with the per cent cardiac ventricular functional recovery rate to determine if a relationship exists amongst this cardiovascular parameter during periods of cardiac oxidative stress.

Keywords: *Inosine, biomarker, mouse cardiac ischaemia, cardiac oxidative stress, high-performance liquid chromatography (HPLC)*

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Introduction

Cardiovascular disease (e.g. myocardial infarction) is one of the leading causes of mortality in the world (Dorner & Rieder 2004, Okrainec et al. 2004, Naudziunas et al. 2005). Current medical evaluation of patients suspected of having a myocardial infarction includes an electrocardiogram blood evaluation for specific biomarkers of cardiac ischaemia and where available radioisotope perfusion studies (Lees 2000,

Correspondence: H. T. Karnes, Department of Pharmaceutics, Virginia Commonwealth University, MCV Station Box 980533, Richmond, VA 23298-0533, USA. Tel: 1-804-828-3819. Fax: 1-804-828-8359. E-mail: htkarnes@vcu.edu

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Beyerle 2002, ADAM, Inc. 2005). Blood evaluation determines the levels of several specific endogenous protein biomarkers (e.g. troponin T, troponin I, creatine kinase MB (CK-MB) and myoglobin); however, these biomarkers are normally indicative of cardiac tissue necrosis and are detected hours after the cardiac ischaemic event and not at the time of initial cardiac ischaemia, which may include angina (stable or unstable but non-necrotic). Ideally, emergency medical services would benefit from a biomarker of early cardiac ischaemia to guide initial treatment and subsequent diagnostic steps in the chest pain patient. Medical conditions (e.g. anxiety attacks, acid reflux and angina) other than myocardial infarction that cause patient chest pain and other constitutional symptoms that might be seen as being consistent with myocardial ischaemia.

To perform its circulatory function, the heart is highly energy dependent on adenosine triphosphate (ATP), which is made in cardiac cellular mitochondria by either aerobic (oxidative phosphorylation via electron transport chain) or anaerobic (glycolysis) processes. The aerobic process is heavily oxygen dependent and generates approximately 80% of cardiac cellular ATP. The anaerobic process is independent of oxygen and produces approximately 20% of the cardiac cellular ATP. Lactic acid is a by-product of anaerobic ATP production.

To produce large quantities of ATP, human cardiac cells have an abundance of mitochondria that comprise approximately 40–50% of the cardiac cellular mass. When cardiac tissue is subjected to periods of constant oxidative stress (e.g. cardiac ischaemia), insufficient oxygen is available for cardiac mitochondria to synthesize aerobically the ATP required for normal cardiac function. This causes a cellular accumulation of ATP metabolic by-products (e.g. adenosine diphosphate (ADP), adenosine monophosphate (AMP)) and activates normally dormant enzymes (e.g. 5′-nucleotidase, adenosine deaminase, purine nucleoside phosphorylase and xanthine oxidase) to catabolize the ATP by-products to substances such as adenosine, inosine, hypoxanthine, xanthine and uric acid for cardiac cellular elimination (Abd-Elfattah et al. 2001). In human cardiac tissue, another source of ATP metabolic by-products is through metabolism of diadenosine polyphosphates, which are released from cardiac-specific secretory granules during periods of cardiac metabolic or ischaemic stress to provide cellular protective functions (Luo et al. 2004).

Inosine (9-β-D-ribofuranosylhypoxanthine) is an endogenous purine nucleoside normally found in the human body as a degradation component of purine metabolism. In human plasma, inosine is metabolized in red blood cells with a reported half-life of <5 min with endogenous plasma levels found in trace amounts (e.g. low ng ml^{-1}) (Viegas et al. 2000). In humans, nature has provided a cellular biochemical mechanism to help conserve energy in producing the required large quantities of ATP for cardiac cellular use (called the salvage pathway), which can convert cellular inosine back to ATP via several enzymatic steps; thus, recycling cellular inosine (Nelson & Cox 2000). However, in periods of constant cardiac oxidative stress (e.g. 20 min), cardiac cells build up significant amounts of ATP metabolic by-products, which activate normally dormant enzymes to catabolize ATP by-products, which then become systemically available before their elimination.

A recently published scientific editorial requested the need for an initial biomarker for cardiac ischaemia before cardiac tissue necrosis (cardiac proteins found in plasma after several hours of cardiac ischaemia) (Morrow et al. 2003). This initial biomarker would aid Emergency Medical Services (EMS) personnel in the rapid treatment of

initial cardiac ischaemia (potentially myocardial infarction), thus potentially increasing the survival rate of myocardial infarction victims every year. One recent publication (Bhagavan et al. 2003) addressing the scientific editorial request describes a blood measurement for serum albumin that appears at an elevated level in the blood in patients undergoing myocardial infarction. However, the authors state that the colorimetric method would not discriminate between ischaemic patients with and without myocardial infarction, thus the need for a method to detect the initial cardiac ischaemic event before myocardial infarction would be beneficial to EMS personnel.

It is the present authors' hypothesis that even before extracellular biomarkers (e.g. serum albumin) appearing in the blood from cardiac ischaemic events, plasma inosine levels would be elevated significantly above the normally low endogenous levels thus becoming a useful biomarker of pre-necrosis cardiac ischaemia. Adenosine, another nucleoside metabolic by-product of ATP catabolism, is metabolized by red blood cells and has a very short plasma half-life (e.g. approximately 15 s); thus making it more difficult to measure it quantitatively in plasma (Mei et al. 1996). The three other metabolic by-products (xanthine, hypoxanthine and uric acid) are normally found at higher levels in the plasma but would lack the necessary specificity due to potential contributions from other human disease state conditions (e.g. plasma uric acid levels elevated in gout; plasma xanthine levels elevated in xanthine oxidase deficient individuals).

The Institute of Cancer Research (ICR) outbred mouse (Dohm 2004) was used as the animal model for all global cardiac ischaemia experiments using a Langendorff apparatus (Xi et al. 1998). For sample analysis, a high-performance liquid chromatographic diode array detection (HPLC-DAD) method was utilized consisting of direct injection of the Krebs buffer eluant from surgically removed and perfused mouse heart tissue. In addition, the HPLC-DAD method utilized current column technology (hydrophobic/hydrophilic reverse-phased retention), which provided sufficient component resolution and sensitivity for adenosine, inosine and xanthine-like derivatives. The HPLC-DAD results were used to compute the inosine area under the concentration (AUC)–time curve from mouse Krebs buffer eluant samples and compared with the per cent cardiac ventricular functional recovery rate to determine if a relationship exist between this cardiovascular parameter during periods of constant cardiac oxidative stress.

Materials and methods

Chemicals, mobile phase and Krebs buffer solution

Hypoxanthine and xanthine were purchased from Acros Organics (Fair Lawn, NJ, USA). 2,3-Dihydroxybenzoic acid (DHBA), 2,5-dihydroxybenzoic acid, salicylic acid (SA), adensosine, inosine and uric acid were purchased from Sigma-Aldrich (St Louis, MO, USA). Sodium chloride, sodium bicarbonate, potassium chloride, magnesium sulfate, monobasic potassium dihydrogen phosphate, dextrose and calcium chloride were used to prepare the Krebs buffer solution, and all were purchased from Sigma-Aldrich. All purchased chemicals were ACS reagent grade or better. The Krebs buffer solution (118.5 mM NaCl, 25.0 mM $NaHCO_3$, 11.1 mM $C_6H_6O_6$, 4.7 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$ and 2.5 mM $CaCl_2$) was prepared in ultrapure deionized water at pH 7.4 and with a 95%O_2:5%CO_2 ratio. For mobile phase preparation, trifluoroacetic acid (TFA) was reagent grade and methanol

was Optima HPLC-grade; both were purchased from Fisher Scientific (Fair Lawn, NJ, USA). Ultrapure distilled and deionized water (18 megaohm) used for HPLC work was prepared in-house using the Purelab Ultra deionized water system (US Filter, Lowell, MA, USA) and filtered before use.

Preparation of standard solutions

Stock standards of adenosine, inosine, hypoxanthine, xanthine and uric acid (100 μg ml^{-1}) were prepared in deionized water and stored at 4°C. Working standards of each component were prepared at 2.5 μg ml^{-1} in Krebs buffer solution and maintained at −20°C along with the mouse Krebs buffer eluant samples. The working standards stored at −20°C were stable for at least 6 months.

HPLC-DAD, high-performance liquid chromatography-mass spectrometry (HPLC-MS) equipment and conditions

For inosine quantification and diode array spectral purity, the HPLC equipment consisted of an Agilent Model 1100 Quaternary HPLC-DAD and Chemstation software (Palo Alto, CA, USA). The DAD was set to acquire a complete ultraviolet light spectrum for component specificity with 240 nm used for quantification of inosine and the other ATP metabolic by-products. For inosine confirmation, liquid chromatography/mass spectrometry (LC/MS) was used and the equipment consisted of a Shimadzu LCMS-2010A HPLC coupled to a single quadrapole mass spectrometer using LCMS Solutions software (Columbia, MD, USA). The HPLC-MS conditions consisted of using electrospray ionization (ESI) with the following instrument set points (heating block at 300°C, nebulizer at 4 l min^{-1} nitrogen, interface voltage at 2 kV) and full-scan acquisition using a positive-ion mode.

The analytical column for both HPLC-DAD and HPLC-MS analysis was a Synergi™ Hydro-RP C_{18}, 150 × 3 mm i.d., 4 μm packing, 80 Å (Phenomenex, Torrance, CA, USA). The C_{18} guard column was a 30 × 4.6 mm i.d., 40–50 μm pellicular packing (Alltech, Deerfield, IL, USA). The mobile phase consisted of aqueous trifluoroacetic acid (0.05% TFA in deionized water, v/v, and pH 2.2) and methanol gradient. The mobile phase gradient was linear with a time-course as follows (95:5 0.05% TFA in deionized water:methanol, v/v at 0 min; 70:30 0.05% TFA in deionized water:methanol, v/v at 12 min; 10:90 0.05% TFA in deionized water:-methanol, v/v at 13 min and held 3 min, and 95:5 0.05% TFA in deionized water:methanol, v/v at 17 min).

The mobile phase was degassed automatically using an Agilent 1100 membrane degasser with a flow-rate of 0.6 ml min^{-1}. An injection volume of 100 μl of the Krebs buffer eluant was made using an autosampler. The typical HPLC operating pressure was approximately 150 bar with ambient column oven temperature and 345 kPa back-pressure regulator (SSI, State College, PA, USA) to prevent mobile phase outgassing in the detector.

ICR mouse experiment conditions

ICR mice were used for all cardiac ischaemia experiments with morphometric characteristics and baseline cardiac function of the adult mice (ICR strain) provided in Table I. The mice were anaesthetized; hearts were surgically removed and isolated

Table I. Morphometric characteristics and baseline cardiac function of the adult mice (ICR strain).

	Control ($n=6$)	Ischemia–reperfusion test ($n=6$)
Body weight (g)	42.2±1.3	38.7±2.1
Heart wet weight (mg)	258±6	242±14
Heart rate (beats per minute, bpm)	368±23	345±23
Developed force (g)	0.81±0.19	1.12±0.12
Rate–force product (g×bpm)	308±80	372±49
Coronary flow (ml min^{-1})	2.3±0.2	1.7±0.1

Values are the mean±standard error of the mean (SEM). No significant difference ($p>0.05$) between the groups was found for the listed parameters, except coronary flow.

using the Langendorff apparatus. Global cardiac oxidative stress was accomplished by adjusting the Krebs-buffered solution to zero flow through the heart for 20 min. Upon heart reperfusion, approximately 1.5-ml samples of Krebs-buffered eluant from the isolated mouse hearts were collected at predetermined time-points (0, 1, 3, 5, 10 and 20 min) in plastic bullet centrifuge tubes and frozen at −20°C until HPLC-DAD analysis.

To evaluate the effects of oxidative stress on the mouse heart, established cardiovascular measurements (e.g. ventricular functional recovery) were performed on both control and test animals. The methodology used to evaluate the isolated perfused mouse heart has been previously described (Xi et al. 1998). In brief, animals were anaesthetized with an intraperitoneal injection of pentobarbital sodium (100 mg kg^{-1}, with 33 IU heparin added). The heart was removed and immediately placed in ice-cold Krebs buffer. The aorta was cannulated within 3 min onto the Langendorff perfusion system and the heart was perfused in a retrograde fashion at a constant pressure of 55 mmHg with Krebs buffer gassed with 95%O_2 and 5% CO_2. The pH of the buffer and the heart temperature were maintained at 7.35–7.50 and 37±0.5°C, respectively. A force-displacement transducer (Grass, FT03) was attached to the apex via a metal hook/surgical thread/pulley system to record and measure the ventricular contractile force and heart rate continuously. For each heart the resting tension was set at approximately 0.3 g in the beginning of the experiment.

The protocol for the test group consisted of 30 min of stabilization, 20 min of zero-flow global ischaemia, and 30 min of reperfusion (Xi et al. 1998). Time-matched normoxic perfusion was carried out for the control group. At the end of each experiment, the heart was removed from the Langendorff system, quickly weighed and stored at −20°C.

Sample preparation, stability and instrument precision evaluation

Before HPLC analysis, perfusate samples frozen at −20°C were thawed to ambient temperature, mixed thoroughly by inversion and transferred to plastic autosampler vials for subsequent direct injection into the HPLC-DAD system. To evaluate sample stability in the perfusate solution and instrument precision; prepared samples in autosampler vials were stored at ambient laboratory temperature overnight and re-injected ($n=3$ times) into the HPLC for analysis.

Component retention times, inosine calibration and AUC calculations

During HPLC method development and validation, combined standards of adenosine, inosine, hypoxanthine, xanthine and uric acid were prepared in Krebs buffer solution at concentration levels of 1, 2.5, 5, 10 and 25 μg ml^{-1}. Standard curve linearity (non-weighted) of all components was acceptable with all correlation coefficients >0.995. During subsequent analytical runs, a single point calibration standard mixture containing 2.5 μg ml^{-1} of each component was prepared in Krebs buffer solution and was used to identify component retention times and the quantification of inosine found in test samples. Using ultraviolet light detection at 240 nm, component peak area and external standardization were used for inosine computations. To determine inosine AUC on the test samples, the trapezoidal rule computation using Excel software was performed on inosine sample values from 0 to 20 min.

Results and discussion

Initial evaluation for (•OH) free radicals

During periods of cardiac oxidative stress (e.g. acute myocardial infarction), the heart is deprived of the oxygen needed for ATP synthesis. In the absence of oxygen, dormant enzymes activate whereby ATP is sequentially converted to ADP, AMP, adenosine, inosine and hypoxanthine. Upon reperfusion of the heart with oxygenated blood or oxygenated Krebs solution, additional cellular enzymatic conversions transpire with the xanthine oxidase converting hypoxanthine to xanthine and uric acid. A metabolic by-product of xanthine oxidase is the formation of hydrogen peroxide (H_2O_2), which is normally converted by glutathione peroxidase to H_2O. However, in the presence of Fe^{2+}, H_2O_2 may be converted to a hydroxyl free radical (•OH) via the Fenton and Haber–Weiss reactions (Figure 1) (IUPAC 1997).

The (•OH) is a known potent reactive oxygen species (ROS) and can cause damage to cellular components (e.g. lipids, proteins, nucleic acids) (Tardif & Bourassa 2000). To investigate the formation of ROS, one research objective was to evaluate and estimate the amount of (•OH) generated from 20 min of global cardiac ischaemia using isolated mouse hearts. In several of the initial experiments, SA (1 mM) was fortified in the Krebs buffer solution (pH adjusted 7.4) to react with (•OH) and form the reaction products of 2,3- and 2,5-DHBA isomers (Onodera & Ashraf 1991, Coudray & Favier 2000). The HPLC-DAD conditions that were used for inosine determination resolved prepared standards (13 ng ml^{-1} or 86 nM) of the 2,3- and 2,5-DHBA isomers from other Krebs eluant sample components (e.g. SA, adenosine, inosine, hypoxanthine, etc.).

However, in the experiments performed using SA we did not observe either the 2,3- or 2,5-DHBA isomers in the sample chromatograms from mouse hearts subjected to global cardiac ischaemia. It is possible that the initial level of SA (1 mM) added to the Krebs buffer solution increased the total solute concentration to a level, which reduced the solubility of 2,3- and 2,5-DHBA isomers and therefore made each analytically undetectable. Lower concentrations of SA (e.g. ≤ 1 μM) may in theory resolve this aspect of ROS generation from mouse global cardiac ischaemia.

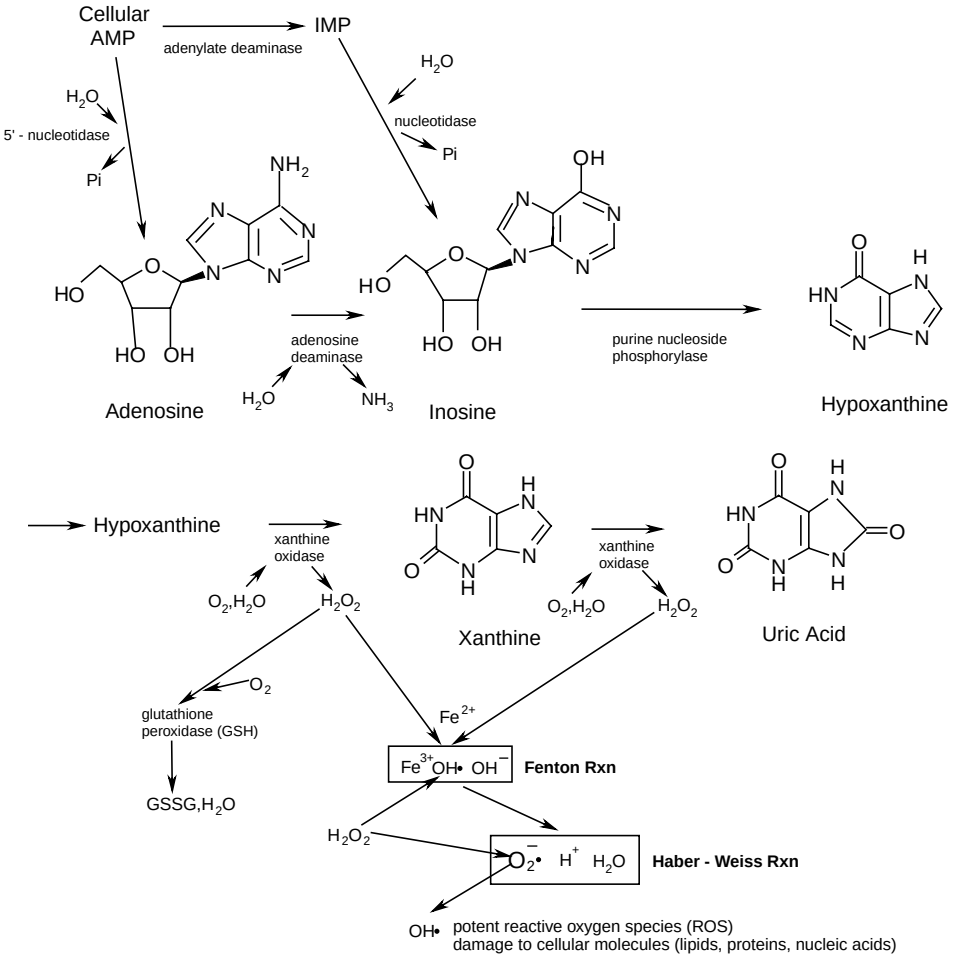

Figure 1. Schematic drawing of cardiac cellular adenosine triphosphate (ATP) catabolism due to oxidative stress and potential oxygen reperfusion injury due to (•OH) free radical generation.

HPLC-DAD and HPLC-MS confirmation

The HPLC-DAD method was used for determining all of the following components (adenosine, inosine, hypoxanthine, xanthine, uric acid, 2,3- and 2,5-DHBA isomers). The mobile phase aqueous component 0.05% TFA in deionized water was chosen as a pH of approximately 2.3 provided good peak shapes on all components and a low pH was necessary to reduce peak tailing on the acidic components (e.g. 2,5-DHBA has a $pK_a \approx 2.9$). The Synergi™ Hydro-RP C_{18} (polar endcapped) and Synergi™ Polar-RP C_{18} (ether-linked phenyl) columns of identical dimensions were evaluated for use. While both columns worked well for inosine and polar components (e.g. adenosine), the Synergi™ Hydro-RP C_{18} was selected for overall analysis as it provided good component peak shape and sufficient resolution of all components.

Other components evaluated using this method have HPLC retention times as follows: uric acid, 2.8 min; hypoxanthine, 3.9 min; xanthine, 4.2 min; adenosine, 5.7 min; CK-MB, 8.2 min; 2,3-DHBA, 8.4 min; 2,5-DHBA, 10.2 min; myoglobin,

14.1 min; atrial natriuretic peptide, 14.5 min; brain natriuretic peptide, 15.0 min; and salicylic acid, 15.4 min. Both troponin I and troponin T were not detected using this HPLC method. An HPLC-DAD chromatogram overlay from a mouse subjected to 20-min global cardiac ischaemia and a control mouse (non-ischaemia) are presented in Figure 2 with inosine elution at 5.9 min.

To evaluate perfusate sample stability, the prepared samples were initially injected and analysed by HPLC-DAD. The samples were subsequently stored overnight on the autosampler at ambient laboratory temperature and re-injected ($n = 3$ times) to evaluate both for changes in component levels due to possible synthesis or degradation reactions from potential enzymes eluted in the perfusate and to evaluate instrument precision. In all re-injected perfusate samples, component levels remained constant ($\leq 4\%$ RSD) indicating stability overnight at ambient temperature and the absence of appreciable levels of nucleoside and purine converting enzymes in the perfusate.

HPLC-MS confirmation of inosine as potential initial ischaemia biomarker

An HPLC-MS was used to confirm inosine at retention time 5.9 min in samples from test mice subjected to oxidative stress. The HPLC analytical column, mobile phase gradient and flow rate were identical to that used in the HPLC-DAD method. The mass spectrum for inosine (MW = 268 Da) is presented in Figure 2. It was acquired using the MS positive-ion mode, which provided a good mass spectral quality match against a prepared standard of inosine in Krebs buffer solution. The full-scan spectrum was achieved using up-front collision-induced dissociation (CID) and nitrogen as the collision gas. The mass spectrum base peak (137 Da) represents the cleavage of the ribose entity from inosine leaving a protonated hypoxanthine (MW = 136 Da).

Evaluation of inosine AUC and other cardiovascular parameters

Initially, the focus was on identifying cardiac protein or peptide biomarkers (e.g. Atrial Natriuretic Peptide, Brain Natriuretic Peptide, that may be released from ischaemic myocardium; however, in comparison with non-ischaemic mouse hearts only inosine

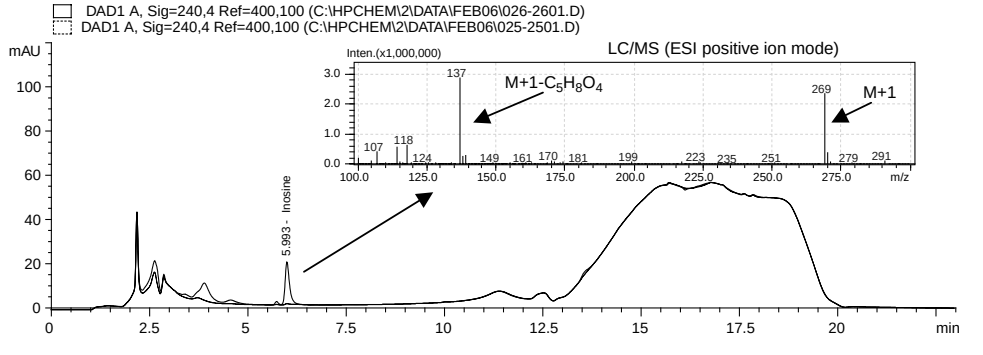

Figure 2. High-performance liquid chromatographic diode array detection (HPLC-DAD) chromatograms overlay of control (025-2501.D) and 20-min global cardiac ischaemia (026-2601.D) mouse perfusate samples. Inosine (retention time = 5.9 min) and high-performance liquid chromatography-electrospray ionization-mass spectrometry (HPLC-ESI/MS) identifying inosine (MW = 268 Da) as a potential early biomarker of global cardiac ischaemia is demonstrated in the ischaemic mouse heart perfusate.

(22–69-fold) and xanthine-like products (e.g. hypoxanthine (>7 ×), xanthine (approximately 3 ×), uric acid (approximately 3 ×)) were found at higher levels in globally ischaemic mouse hearts. Figure 3 is a representative profile of one mouse heart subjected to oxidative stress with the individual ATP degradation by-product components DAD response plotted against Krebs buffer reperfusion time. As can be seen, inosine was the component that had the highest DAD response with detectable component amounts found at lower μg ml^{-1} levels (e.g. in a range of 0.4–7.5 μg ml^{-1} in mouse #874) in the sample less than 5 min following reperfusion.

Other cardiovascular parameters (e.g. the per cent cardiac ventricular functional recovery rate) were measured and reported with the calculated inosine AUC results (Table II). As can be seen in Table II, inosine efflux was present in test mouse heart perfusate samples that were subjected to oxidative stress and was not detected in control mouse heart perfusate samples. However, for both controls and test mice, the per cent cardiac functional recovery rate ranged from 39 to 92%, with the lowest measured cardiac functional recovery being in test mouse hearts that had the largest amount of inosine present in the Krebs buffer solution (e.g. test mouse with 2469 ng min ml^{-1} AUC inosine effluxed with a 39% cardiac functional recovery rate). This may indicate that mouse hearts are injured to a greater degree from the effects of oxidative stress efflux more inosine from ATP by-product degradation. Further studies with larger test and control mouse sample size (e.g. $n = 10$) would be necessary to interpret this observation statistically.

Conclusions

The preliminary results suggest that the level of inosine found in test animals subjected to cardiac oxidative stress may serve as a potential biomarker indicative of early cardiac ischaemia. This can be explained by ischaemic myocytes undergoing nucleotide purine catabolism in the absence of oxygen with subsequent activation of dormant cellular enzymes and the generation of degradative breakdown products of ATP. Preliminary human studies will need to be undertaken to determine the validity of this hypothesis.

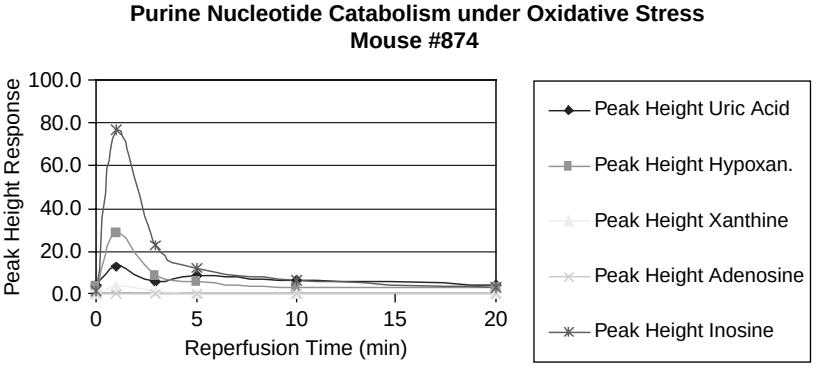

Figure 3. Profile of adenosine triphosphate (ATP) catabolism by-products found in Krebs solution versus reperfusion time after 20-min mouse global cardiac ischaemia. Inosine (μg ml^{-1}) levels were 0, 7.5, 2.1, 1.1, 0.4 and not detected for sample time points 0, 1, 3, 5, 10 and 20 min, respectively.

Table II. Inosine washout and cardiac ventricular functional recovery in Langendorff mouse hearts following aerobic perfusion and 20-min global ischaemia.

Sample type	Inosine area under the curve (AUC) 0–20 min (ng min ml^{-1})	Cardiac Functional recovery rate (%)
Control	n.d.	70
Control	n.d.	72
Control	n.d.	74
Control	n.d.	82
Control	n.d.	81
Control	n.d.	64
Test	653	92
Test	962	84
Test	954	77
Test	1003	53
Test	2469	39
Test	2583	52

n.d., Not detected.

Additional research from the present authors' group will be performed on plasma samples obtained from hospital-admitted patients undergoing myocardial infarction to determine if plasma levels of endogenous inosine are significantly elevated during periods of cardiac oxidative stress. If inosine plasma levels are found to be significantly elevated in samples obtained from patients undergoing myocardial infarction, inosine should be recommended as a potential biomarker for the initial cardiac ischaemic event and may be useful in indicating the need for immediate medical treatment, potentially improving patient outcome.

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