Accumulation of Unsaturated Acylcarnitine Molecular Species During Acute Myocardial Ischemia: Metabolic Compartmentalization of Products of Fatty Acyl Chain Elongation in the Acylcarnitine Pool[†]

David A. Ford,*,[‡] Xianlin Han, Caroline C. Horner, and Richard W. Gross

Division of Bioorganic Chemistry and Molecular Pharmacology, Department of Internal Medicine, Washington University School of Medicine, St. Louis, Missouri 63110

Received March 6, 1996[⊗]

ABSTRACT: Long-chain acylcarnitines accumulate during myocardial ischemia and contribute to membrane dysfunction in ischemic zones. On the basis of the 3-fold selectivity for saturated fatty acid accumulation during myocardial ischemia, it was implicitly assumed that saturated long chain acylcarnitine molecular species predominantly accumulated in ischemic myocardium. By exploiting the analytical power of electrospray ionization mass spectroscopy, we now report that unsaturated acylcarnitines are the predominant molecular species of acylcarnitine which accumulate during myocardial ischemia (rank order: octadecadienoyl carnitine > octadecenoyl carnitine > hexadecanoyl carnitine > octadecanoyl carnitine). The aliphatic chain distribution of myocardial acylcarnitine molecular species identified by electrospray ionization mass spectroscopy was independently substantiated by sequential HPLC purification and capillary gas chromatography. Detailed analysis of the individual molecular species of long-chain acylcarnitine demonstrated that fatty acyl chain elongation was prominent in ischemic myocardium (e.g., following 20 min of ischemia, greater than 15% of the accumulated acylcarnitines consisted of 20-carbon unsaturated molecular species). Chain-elongated lipids were essentially confined to the long chain acylcarnitine pool since [9,10-^{3}H]octadec-9′-enoic acid was converted to [^{3}H]eicosenoyl carnitine (12% of the radiolabeled acylcarnitine pool) in ischemic hearts without substantive amounts of [^{3}H]eicosenoyl residues in the fatty acid, triglyceride, and phospholipid pools. Collectively, these results demonstrate the preponderance of unsaturated acylcarnitines in ischemic myocardium and document the metabolic compartmentation of downstream products of fatty acyl chain elongation in the acylcarnitine pool during ischemia.

Long-chain acylcarnitines have been implicated as potential biochemical mediators of the pathophysiologic sequelae of myocardial ischemia (Whitmer et al., 1978; Idell-Wenger et al., 1978; Liedtke & Nellis, 1979; Corr et al., 1981, 1984). Due to the predominant accumulation of saturated fatty acids in ischemic myocardium (Weishaar et al., 1977; Chien et al., 1984; Ford & Gross, 1989), it was previously assumed that the acylcarnitine molecular species which accumulate in ischemic myocardium were composed of saturated aliphatic species. Accordingly, experiments from multiple laboratories have examined the potential pathophysiologic significance of long-chain acylcarnitines on myocardial function utilizing hexadecanoyl carnitine as a model compound [e.g., Corr et al. (1981, 1984) and Sato et al. (1993)]. However, identification of the mechanism through which acylcarnitines exert their deleterious effects on myocardial function utilizing hexadecanoyl carnitine as a model compound has not been forthcoming.

Since myocardial ischemia is accompanied by dramatic increases in the mass of the acylcarnitine pool and since the biologic sequelae of amphiphilic constituents containing unsaturated aliphatic chains can be quite different from their saturated counterparts in some systems [e.g., Okamura and Yamashita (1994), Cao and Hatch (1994), and Chung and Fleming (1995)], we sought to identify the distribution of individual molecular species of long-chain acylcarnitines in ischemic myocardium. Historically, the mass of carnitines in biologic tissues has been quantitated by measuring the carnitine acetyltransferase catalyzed conversion of tissue carnitine to [^{3}H]acetyl carnitine (McGarry & Foster, 1976). To specifically assess long-chain acylcarnitine content, tissue homogenates are subjected to sequential perchloric acid precipitation and base-catalyzed hydrolysis to provide the carnitine substrate for the carnitine acetyltransferase reaction (McGarry & Foster, 1976). Although this methodology has faithfully allowed analyses of total long-chain acylcarnitine mass, it cannot assess alterations in individual molecular species of long-chain acylcarnitines and therefore is unsuitable for the measurement of the flux of individual fatty acids into and out of the acylcarnitine pool. Our recent mass spectroscopic studies with a structurally similar class of lipids, lysophospholipids (e.g., lysophospholipids and acylcarnitines are each zwitterionic, possess esterified long-chain aliphatic moieties, and contain a quaternary amine) (Gross & Sobel, 1982), suggested that the efficiency inherent in the electrospray ionization approach would facilitate the direct quantitation of the mass of individual molecular species of long-chain acylcarnitines from chloroform extracts of intact tissue (Han & Gross, 1994). Herein we utilize electrospray ionization mass spectroscopy to demonstrate that unsaturated

[†] This research was supported by Grant HL42665 and Research Career Development Award HL03316 (to D.A.F.) from the National Institutes of Health.

* Tel: (314) 577-8123. FAX: (314) 577-8156.

[‡] Present address: Department of Biochemistry and Molecular Biology, St. Louis University Health Sciences Center, St. Louis, MO 63104.

[⊗] Abstract published in *Advance ACS Abstracts,* June 1, 1996.

S0006-2960(96)00552-1 CCC: $12.00

acylcarnitines are the predominant molecular species which accumulate during myocardial ischemia and that the long-chain acylcarnitine pool is a metabolic repository for the products of fatty acyl chain elongation in ischemic myocardium.

EXPERIMENTAL PROCEDURES

Preparation of Langendorff-Perfused Rabbit Hearts, Induction of Myocardial Ischemia, and Tissue Extraction. Rabbit hearts were perfused retrograde via the aorta (Langendorff-perfused) with either modified Krebs−Henseleit buffer consisting of 137 mM NaCl, 4.7 mM KCl, 3 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 0.5 mM NaEDTA, 15 mM $NaHCO_3$, and 11 mM glucose equilibrated with O_2/CO_2 (95/5) or modified Krebs−Henseleit buffer supplemented with 1.2 mM hexadecanoic and 0.3% fatty acid-free bovine serum albumin. Langendorff-perfused hearts were either control-perfused at 60 mmHg (control), rendered globally-ischemic for selected intervals, or rendered globally-ischemic for 5 min followed by reperfusion for 20 min as previously described (Ford & Gross, 1989). In selected studies, Langendorff-perfused hearts were perfused in a recirculating configuration with 500 mL of modified Krebs−Henseleit buffer containing 1.5 mCi of [9,10-^{3}H]octadec-9′-enoic acid (60 Ci/mmol). At the end of each perfusion interval, hearts were rapidly freeze-clamped and myocardial tissue was pulverized to a fine powder at the temperature of liquid nitrogen. Myocardial acylcarnitine molecular species were extracted from ∼0.5 g_{wet} of myocardial tissue by a modified Bligh and Dyer technique utilizing 1% acetic acid in the aqueous layer in the presence of 15 μg of pentadecanoyl carnitine (internal standard) (Bligh & Dyer, 1959).

Electrospray Ionization Mass Spectroscopy of Acylcarnitine. Quantitative analyses of acylcarnitine molecular species by electrospray ionization mass spectroscopy were performed in the positive ion mode utilizing a triple-quadrupole tandem mass spectrometer equipped with an electrospray interface as previously described (Han & Gross, 1994). Tandem mass spectroscopy was performed by passage of the mass-selected precursor ion (windows were ≤2 amu) from the first quadrupole into the collision cell (collision energies of ∼20 eV and a collision pressure of ∼3 mTorr of Ar(g) were employed). Typically, spectra were signal-averaged for 5 min and processed utilizing commercial software (ICIS, Finnigan). Chloroform extracts of myocardial samples were diluted 10-fold in chloroform/methanol (1/2, v/v) prior to their direct infusion into the electrospray ionization chamber (flow rate of 1.5 μL/min). The mass of individual acylcarnitine molecular species in control and globally-ischemic hearts was quantitated by comparisons of the individual peak intensities of molecular ions to that of the corresponding internal standard (pentadecanoyl carnitine) after correction for ^{13}C isotope effects.

Analyses of the Acylcarnitine Molecular Species in Globally Ischemic Rabbit Hearts. Acylcarnitine molecular species from the chloroform extracts of control and globally ischemic rabbit hearts were purified utilizing an Ultrasphere-Si column (4.6 × 250 mm; 5 μm) as the stationary phase with an initial linear gradient over 10 min from a mobile phase comprised of hexane/isopropanol/0.005% acetic acid in water (48.5/48.5/3, v/v) to the same solvents (47.75/47.75/4.5, v/v). After 8 min a step change to hexane/isopropanol/0.005% acetic acid (46.5/46.5/7, v/v) was applied, which was maintained for 30 min. In experiments designed to identify the aliphatic chain composition of myocardial acylcarnitines, the elution of acylcarnitines was determined by the elution profile of tracer amounts of [*carnitine*-^{3}H]hexadecanoyl carnitine. The elution profile of radiolabeled acylcarnitines generated from [9,10-^{3}H]octadec-9′-enoic acid perfusion of hearts was determined by its coelution with synthetic octadecadi-9′,12′-enoyl carnitine that was added for internal calibration. The elution of octadecadi-9′,12′-enoyl carnitine and other lipids during straight-phase HPLC was monitored at 205 nm.

To determine the aliphatic constituents of myocardial acylcarnitines by capillary gas chromatography, straight-phase HPLC-purified acylcarnitines were sequentially hydrolyzed with base (2 N KOH for 60 min) and their released aliphatic constituents were extracted into ether prior to their derivatization to fatty acid methyl esters utilizing methanolic HCl (Gross, 1984). Alternatively, myocardial acylcarnitine molecular species from [9,10-^{3}H]octadec-9′-enoic acid-labeled hearts were extracted by a modified Bligh−Dyer method (1% acetic acid in the aqueous phase) and purified by straight-phase HPLC, and their aliphatic groups were converted to their bromophenacyl esters utilizing dicyclohexyl 18-crown-6 as catalyst prior to their resolution by reversed-phase HPLC (Durst et al., 1975; Püttmann et al., 1993). Briefly, individual molecular species of the bromophenacyl ester derivatives of myocardial acylcarnitines were separated by reversed phase HPLC employing an Ultrasphere-ODS column (4.6 × 250 mm; 5 μm) as the stationary phase and a mobile phase composed of 7% water in methanol at a flow rate of 1.5 mL/min with UV detection at 254 nm. The identity of radiolabeled bromophenacyl ester molecular species was determined by their coelution with authentic standards produced by derivatizing octadec-9′-enoic and eicos-11′-enoic acid to their respective bromophenacyl esters.

Purification and Analyses of the Aliphatic Constituents of Fatty Acid, Triradylglycerol, and Phospholipids of Control-Perfused and Globally-Ischemic Rabbit Hearts. Radiolabeled triradylglycerols (R_f = 0.75) and fatty acids (R_f = 0.45) were purified by thin-layer chromatography utilizing a mobile phase composed of petroleum ether/ethyl ether/acetic acid (70/30/1,v/v). Phosphatidylcholine (R_t = 26 min) and phosphatidylethanolamine (R_t = 6 min) were purified by straight-phase HPLC utilizing an identical stationary phase, mobile phase, and gradient system as that used for the purification of acylcarnitine (*vide supra*) except that water was substituted for 0.005% acetic acid in the mobile phase. To identify radiolabeled acyl constituents of these myocardial lipids, purified lipids were hydrolyzed (2 N KOH for 60 min) and converted to their respective bromophenacyl ester derivatives which were then separated by reversed-phase HPLC as described above.

Synthesis and Purification of Pentadecanoyl Carnitine, Octadecadi-9′,12′-enoyl Carnitine, and [carnitine-^{3}H]Hexadecanoyl Carnitine. Pentadecanoyl carnitine was synthesized by stirring 200 mg of pentadecanoyl chloride, 200 mg of pentadecanoic acid, 56 mg of carnitine chloride, and 170 μL of trifluoroacetic acid together at 55 °C for 12 h (Squire, 1991). Similarly, [*carnitine*-^{3}H]hexadecanoyl carnitine was synthesized by stirring 70 mg of hexadecanoyl chloride, 70 mg of hexadecanoic acid, 500 μCi of [^{3}H]carnitine (60 Ci/

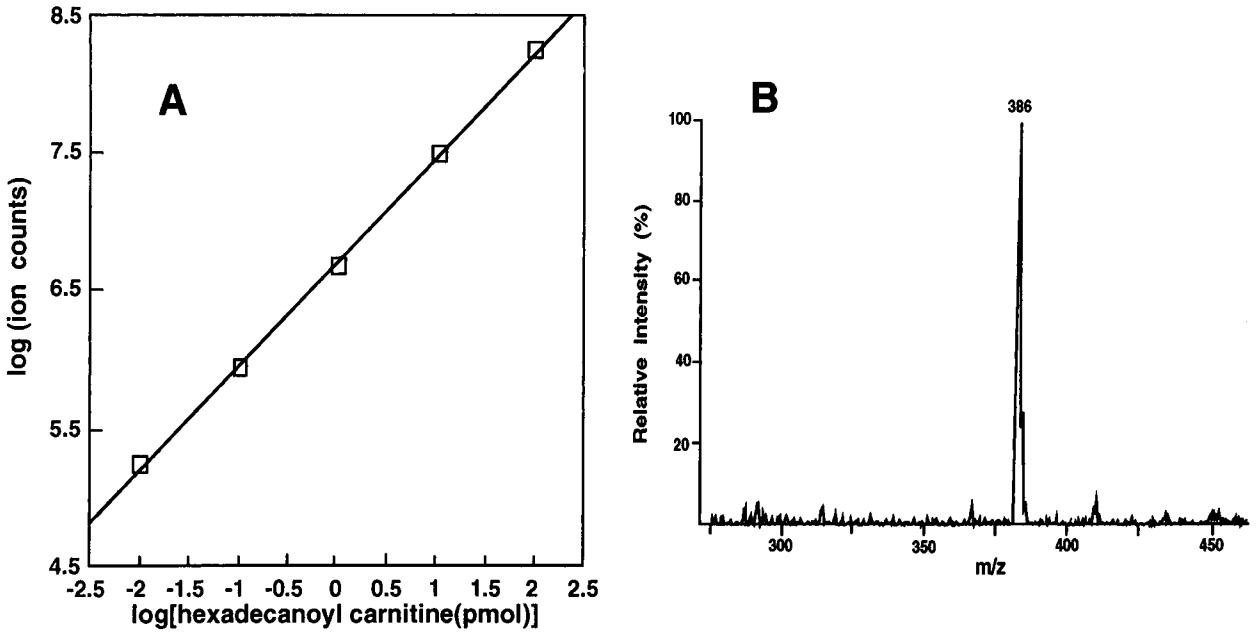

FIGURE 1: Concentration dependence of hexadecanoyl carnitine mass on ion current after electrospray ionization and mass spectrum of synthetic pentadecanoyl carnitine. A double-logarithmic plot of the signal intensity (i.e., ion counts) vs the mass of hexadecanoyl carnitine consumed by electrospray ionization mass spectroscopy (described in detail in Experimental Procedures) yielded a line with a slope of 0.768 and a *y* intercept of 6.732 (panel A). The sensitivity of the electrospray ionization method is illustrated by the response of the detector ($\sim 2 \times 10^6$ ion counts/picomole) and the accuracy is illustrated by the coefficient of correlation ($r^2 > 0.999$). Electrospray ionization mass spectrometry in the positive ion mode was utilized to assess the purity of synthetic pentadecanoyl carnitine (*m*/*z* 386) (panel B) as described in detail in Experimental Procedures.

mmol) (American Radiolabeled Chemicals, St. Louis, MO), and 65 μL of trifluoroacetic acid together at 55 °C for 12 h (Squire, 1991). Octadecadi-9′,12′-enoyl carnitine was synthesized by incubating carnitine perchlorate (produced by mixing 150 mg of carnitine chloride and 180 mg of $AgClO_4$ in 3.75 mL of acetonitrile) with 500 mg of octadecadi-9′,-12′-enoyl chloride and 0.005% butylated hydroxytoluene at 24 °C for 12 h (Christophersen & Bremer, 1972). Pentadecanoyl carnitine, octadecadi-9′,12′-enoyl carnitine, and [*carnitine*-^{3}H]hexadecanoyl carnitine were purified by liquid−liquid partitioning as previously described (Squire, 1991). The purities of pentadecanoyl carnitine and octadecadi-9′,-12′-enoyl carnitine were confirmed to be >99% by electrospray ionization mass spectroscopy. [*carnitine*-^{3}H]hexadecanoyl carnitine was further purified by straight-phase HPLC (*vide supra*).

Materials. Dibromoacetophenone and dicyclohexyl 18-crown-6 were purchased from Aldrich Chemicals. Organic solvents were purchased from Fisher Chemical. All other materials were of the highest grade available from Sigma Chemical.

RESULTS

The selectivity and sensitivity of electrospray ionization mass spectroscopy were exploited to identify individual molecular species of acylcarnitine in control and globally-ischemic myocardium. First, the anticipated utility of electrospray ionization mass spectroscopy in the positive ion mode for analysis of acylcarnitine molecular species was corroborated by demonstration of a linear relationship between hexadecanoyl carnitine mass and ion current over a 10 000-fold dynamic range (between 0.01 and 100 pmol) with a correlation coefficient (r^2) > 0.999 (Figure 1A). Next, an internal standard, pentadecanoyl carnitine, was synthesized and purified as described in Experimental Procedures, and its purity was confirmed by electrospray ionization mass spectroscopy (Figure 1B). Since pentadecanoyl carnitine is not an endogenous metabolite in myocardium, it represents a suitable internal standard for quantitation of long-chain acylcarnitine content from mass spectra.

Mass spectroscopic analyses of acylcarnitine molecular species from rabbit hearts perfused with modified Krebs−Henseleit buffer prior to 5 min of global ischemia demonstrated the unanticipated abundance of unsaturated acylcarnitine molecular species. Collectively, unsaturated acylcarnitine molecular species increased 2.5-fold more than their saturated counterparts following both 5 and 20 min of global ischemia [rank order: octadecadienoyl (*m*/*z* 424) > octadecenoyl (*m*/*z* 426) > hexadecanoyl (*m*/*z* 400) > octadecanoyl carnitine (*m*/*z* 428)] (Figure 2, Table 1). Furthermore, 20 min of global ischemia resulted in the accumulation of substantial amounts of eicosenoyl (*m*/*z* 454) and eicosadienoyl (*m*/*z* 452) acylcarnitine molecular species, which represented 15% of the increase in total acylcarnitine accumulation manifest in ischemic tissue (Figure 2C, Table 1). The identity of each molecular ion corresponding to individual acylcarnitine molecular species was confirmed by tandem mass spectroscopy by mass selection and subsequent collisional activation of each molecular ion and demonstration of product ions at *m*/*z* 85 (i.e., carnitine) and the respective fatty acid in each case. The accumulation of these long-chain acylcarnitine molecular species during ischemia was reversible since reperfusion of ischemic hearts resulted in the return of the mass of each acylcarnitine molecular species to control levels (Table 1). Finally, even in the presence of supraphysiologic amounts of exogenously-supplied hexadecanoic acid in the perfusate, unsaturated acylcarnitines still represented ~35% of the total acylcarnitine pool which was accompanied by the anticipated increase in hexadecanoyl carnitine (Table 1).

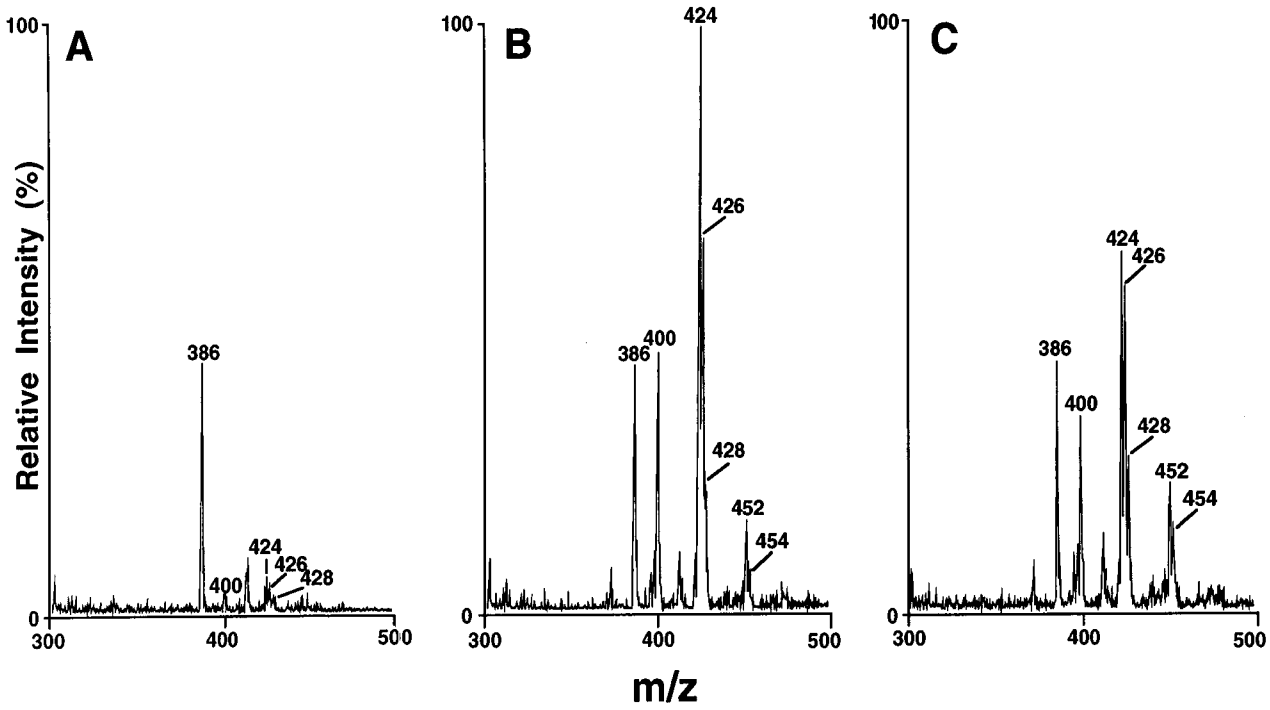

FIGURE 2: Identification and quantitation of the acylcarnitine molecular species in control-perfused and globally-ischemic rabbit myocardium utilizing electrospray ionization mass spectroscopy. Acylcarnitine molecular species from rabbit hearts subjected to control perfusion (panel A), 5 min of global ischemia (panel B), or 20 min of global ischemia (panel C) were extracted from freeze-clamped myocardium (0.5 g) as described in Experimental Procedures. Acylcarnitine molecular species were subsequently quantitated by electrospray ionization mass spectroscopy in the positive ion mode as their protonated molecular ions [pentadecanoyl (internal standard), *m*/*z* 386; hexadecanoyl, *m*/*z* 400; octadecadienoyl, *m*/*z* 424; octadecenoyl, *m*/*z* 426; octadecanoyl, *m*/*z* 428; eicosadienoyl, *m*/*z* 452; and eicosenoyl, *m*/*z* 454]. The contents of each endogenous acylcarnitine molecular species under each of the conditions studied are directly comparable since spectra are normalized to the intensity of the pentadecanoyl carnitine peak (*m*/*z* 386) and the same mass of tissue has been extracted.

Table 1: Quantitation of Individual Acylcarnitine Molecular Species in Control, Ischemic, and Reperfused Langendorff-Perfused Rabbit Hearts[a]

group	14:0	16:0	18:0	18:1	18:2	20:1	20:2	20:4	total
				Palmitate-Free Perfusion Buffer					
control	30 ± 10	65 ± 16	61 ± 21	73 ± 11	94 ± 13	20 ± 8	22 ± 14	31 ± 1	396 ± 81
5 min-ischemic	89 ± 12	700 ± 125	311 ± 29	871 ± 96	1349 ± 73	73 ± 29	148 ± 42	74 ± 25	3615 ± 26
20 min-ischemic	81 ± 17	435 ± 10	290 ± 28	589 ± 87	689 ± 94	130 ± 22	248 ± 5	38 ± 10	2500 ± 52
reperfused	22 ± 8	40 ± 2	33 ± 7	52 ± 5	52 ± 8	10 ± 5	14 ± 8	9 ± 3	232 ± 20
				Palmitate-Supplemented Perfusion Buffer					
control	40 ± 5	210 ± 33	46 ± 11	56 ± 23	47 ± 28	12 ± 2	17 ± 9	11 ± 5	439 ± 99
20 min-ischemic	110 ± 17	705 ± 153	241 ± 78	253 ± 63	325 ± 15	51 ± 12	91 ± 24	17 ± 1	1793 ± 451

[a] Individual molecular species of acylcarnitine were identified and quantitated from perfused (control), globally-ischemic (ischemic), and reperfused globally-ischemic (reperfused) rabbit heart as described in Experimental Procedures. Each value represents the mean of at least three determinations ± SEM. The values are in nmol/g_{dry}.

To substantiate the conclusions from mass spectroscopic analyses of acylcarnitine molecular species in ischemic myocardium, the results were independently confirmed by HPLC purification of acylcarnitine from chloroform extracts of myocardium and analysis of the aliphatic chain distribution of acylcarnitines by capillary gas chromatography. Utilizing the straight-phase HPLC system described in Experimental Procedures, acylcarnitines elute at 35 min [after phosphatidylcholine and sphingomyelin (R_t = 26 and 27 min, respectively) and before lysophosphatidylcholine (R_t = 43 min)] (Figure 3). Tracer amounts of [*carnitine*-^{3}H]hexadecanoyl carnitine were added to chloroform extracts of myocardial homogenates to serve as an internal calibration for the elution of acylcarnitine molecular species. Fractions containing radioactivity were pooled, hydrolyzed (which removed the [^{3}H]carnitine]), and subsequently converted to their fatty acid methyl ester derivatives. Capillary gas chromatography of these fatty acid methyl ester derivatives demonstrated a nearly identical aliphatic chain distribution of HPLC-purified myocardial acylcarnitine to that determined by electrospray ionization mass spectroscopy [i.e., enrichment of octadecadienoyl and octadecenoyl carnitine during 5 min of global ischemia (Figure 4B) and the appearance of eicosadienoyl and eicosenoyl carnitine during prolonged intervals of ischemia (Figure 4C)]. Additionally, it should be appreciated that the total long-chain acylcarnitine mass present in ischemic myocardium as quantitated directly from electrospray ionization mass spectroscopy was similar to that previously reported utilizing the carnitine acetyltransferase technique after sequential perchloric acid precipitation of long-chain acylcarnitines from myocardial homogenates, base-catalyzed release of carnitine, and enzymatic assay (Whitmer et al., 1978; Idell-Wenger et al., 1978; Liedtke & Nellis, 1979; Corr et al., 1981, 1984).

To identify the biochemical mechanism underlying the unanticipated accumulation of 20-carbon molecular species

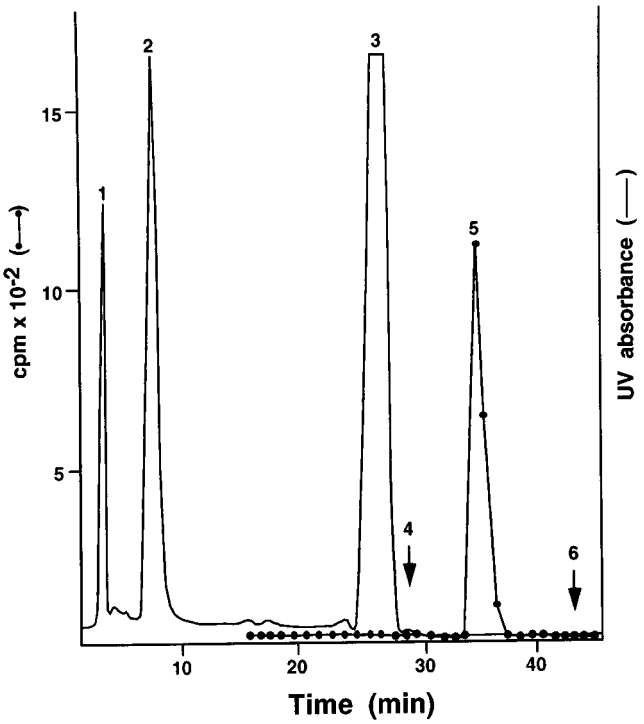

FIGURE 3: HPLC purification of myocardial acylcarnitine. Acylcarnitine was extracted from freeze-clamped myocardium (0.5 g) by a modified Bligh−Dyer technique (Bligh & Dyer, 1959) as described in Experimental Procedures. Acylcarnitine was purified from the chloroform extract utilizing an Ultrasphere silica HPLC column (4.5 × 250 mm; 5 μm; Beckman) by gradient elution at a flow rate of 2 mL/min as described in Experimental Procedures. The elution of neutral lipids (1), phosphatidylethanolamine (2), and phosphatidylcholine (3) was monitored by UV detection at 205 nm (—). The elution profile of acylcarnitine (5) was determined utilizing tracer amounts (i.e., 0.1 μCi) of [*carnitine*-^{3}H]palmitoyl carnitine as an internal calibration (•). The retention times of sphingomyelin (4) and lysophosphatidylcholine (6) were determined in independent chromatographies that included the co-injection of sphingomyelin, lysoplasmenylcholine, phosphatidylcholine, and acylcarnitine.

of acylcarnitine, Langendorff-perfused hearts were prelabeled with [9,10-^{3}H]octadec-9′-enoic acid prior to the onset of global ischemia. Long-chain acylcarnitines were purified by straight-phase HPLC, and analyses of their radiolabeled aliphatic chains were quantitated by reversed-phase HPLC of their bromophenacyl ester derivatives. Ischemic rabbit hearts contained substantial amounts of radiolabeled eicosenoyl carnitine (12% of the radiolabeled acylcarnitine pool) (Figure 5B) while virtually no radiolabeled eicosenoyl carnitine was detected in the control-perfused hearts (Figure 5A). Furthermore, less than 1% of the radiolabel in triradylglycerol, phosphatidylcholine, phosphatidylethanolamine, and phosphatidylinositol pools was present in [^{3}H]-eicosenoic residues in either control or ischemic [9,10-^{3}H]octadec-9′-enoic acid-labeled hearts (Figure 6). The fatty acid pool from ischemic myocardium contained only 1.7% of the radiolabel originating from [9,10-^{3}H]octadec-9′-enoic acid as [^{3}H]eicosenoic acid (Figure 6). The ischemia-induced flux of [9,10-^{3}H]octadec-9′-enoic acid into eicosenoyl carnitine and the progressive accumulation of both eicosenoyl and eicosadienoyl carnitine mass following 5 and 20 min of global ischemia collectively demonstrate fatty acyl chain elongation during myocardial ischemia with specific metabolic compartmentalization of downstream intermediates of fatty acid metabolism in the acylcarnitine pool.

DISCUSSION

Prior analyses of alterations in acylcarnitine content during pathophysiologic perturbations have been limited to quantitation of total acylcarnitine mass since prior methodologic difficulties have precluded measurement of the individual molecular species of acylcarnitine (McGarry & Foster, 1976). Due to the predominant accumulation of saturated fatty acids during brief global ischemia (Weishaar et al., 1977; Chien et al., 1984; Ford & Gross, 1989), it had previously been assumed that the molecular species distribution of the acylcarnitine pool during pathophysiologic perturbations (e.g., ischemia) paralleled the individual molecular species distribution of its penultimate metabolic precursor, non-esterified fatty acid (i.e., saturated molecular species). The present results clearly reveal that the acylcarnitine molecular species which accumulate during acute myocardial ischemia are predominantly composed of unsaturated molecular species and that the molecular species distribution of long-chain acylcarnitines does not reflect the individual molecular species composition of its non-esterified fatty acid metabolic precursors (Weishaar et al., 1977; Chien et al., 1984; Ford & Gross, 1989). The sensitivity (subpicomole), specificity (molecular ions can each be confirmed by tandem mass spectroscopy), simplicity (direct injection of chloroform extracts of tissue), and the analytic power (kinetic analysis of stable isotope flux) of electrospray ionization mass spectroscopy in the analysis of acylcarnitine molecular species will clearly facilitate the quantitative dissection of the contribution of each of the distinct metabolic pathways leading to fatty acid utilization during multiple pathophysiologic perturbations (*vide infra*).

The biochemical mechanisms responsible for the accumulation of fatty acids and their metabolites during myocardial ischemia have been the subject of intense controversy for decades. In large part, this debate has focused on the relative contribution of exogenous fatty acid to the contribution of endogenous fatty acid provided by accelerated hydrolysis of intracellular triglycerides during the ischemic process (Zierler, 1976). The present results demonstrate that both exogenously-supplied fatty acid and endogenously-released fatty acids are important contributors to the acylcarnitine pool since increases in both hexadecanoyl carnitine and unsaturated 18-carbon acylcarnitines occur in hearts perfused with Krebs−Henseleit buffer containing hexadecanoic acid and subsequently rendered globally-ischemic. Finally, the analysis of the individual molecular species of acylcarnitine in ischemic myocardium has identified a third quantitatively important pathway (i.e., fatty acyl chain elongation) contributing to the individual molecular species of acylcarnitines that accumulate during ischemia.

Although it has previously been suggested that the increases in NADH and acetyl CoA which occur during myocardial ischemia could lead to long-chain fatty acyl elongation, prior examination of the non-esterified fatty acid, triglyceride, and phospholipid pools in ischemic myocardium did not demonstrate products of fatty acyl chain elongation (Hagve & Sprecher, 1989; Mohammed et al., 1990). Since 20-carbon mono- and dienoic fatty acids are present in only diminutive amounts in myocardial polar and nonpolar lipid pools (Ford & Gross, 1989), the identification of progressive increases in the content of 20-carbon mono- and dienoic acylcarnitine molecular species following 5 and 20 min of

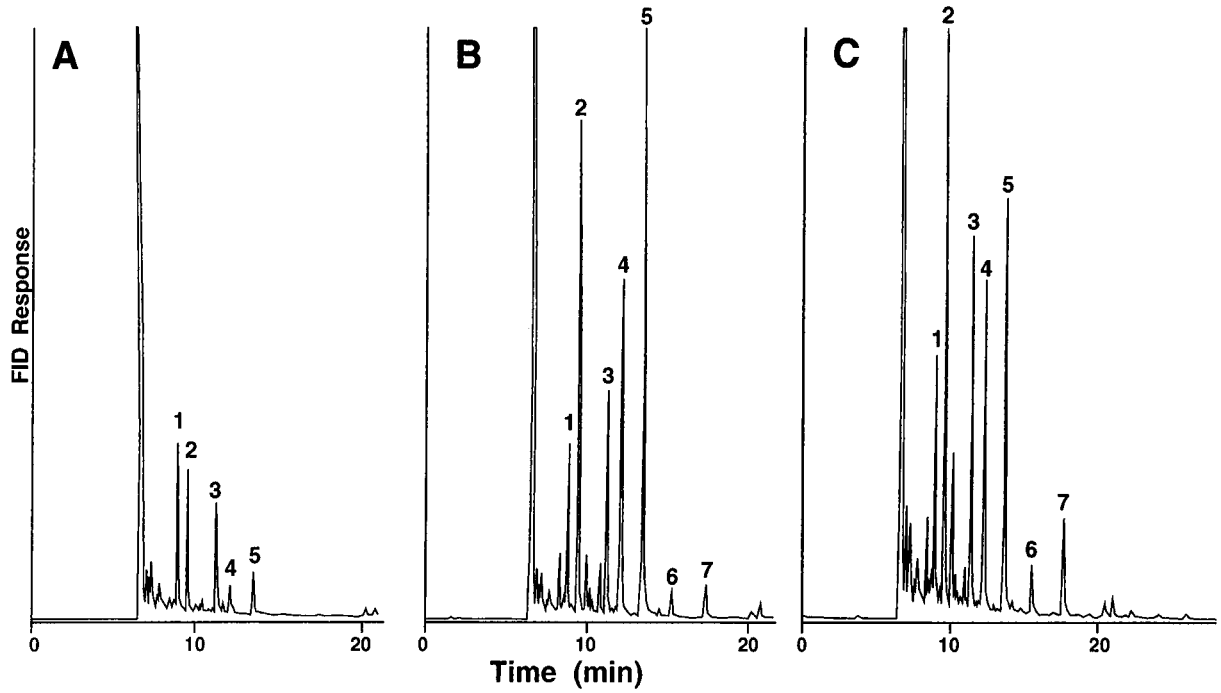

FIGURE 4: Identification and quantitation of the acylcarnitine molecular species in control-perfused and globally-ischemic rabbit myocardium utilizing capillary gas chromatography. Acylcarnitine molecular species from rabbit hearts subjected to control perfusions (panel A), 5 min of global ischemia (panel B), or 20 min of global ischemia (panel C) were independently identified by capillary gas chromatography after HPLC purification and derivatization as described in detail in Experimental Procedures. Peaks were identified as pentadecanoyl methyl ester (1) (internal standard), hexadecanoyl methyl ester (2), octadecanoyl methyl ester (3), octadecenoyl methyl ester (4), octadecadienoyl methyl ester (5), eicosenoyl methyl ester (6), and eicosadienoyl methyl ester (7) by comparisons with authentic fatty acyl methyl esters.

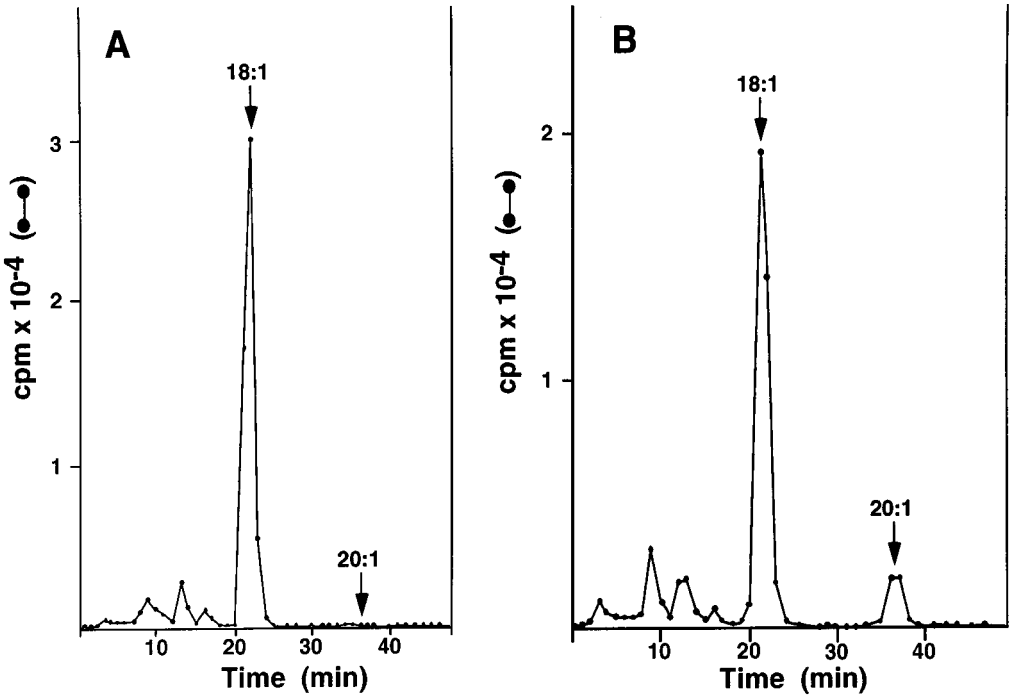

FIGURE 5: Reversed-phase HPLC separation of the bromophenacyl ester derivatives of [^{3}H]acylcarnitine molecular species produced by control and globally-ischemic rabbit myocardium prelabeled with [9,10-^{3}H]octadec-9′-enoic acid. Rabbit hearts were prelabeled with [9,-10-^{3}H]octadec-9′-enoic acid prior to control perfusion (panel A) or global ischemia for 20 min (panel B). Myocardial acylcarnitines were sequentially extracted from freeze-clamped myocardium (0.5 g) by a modified Bligh−Dyer technique (Bligh & Dyer, 1959), purified by straight-phase HPLC and converted to their bromophenacyl ester derivatives. Individual molecular species of bromophenacyl esters were separated by reversed-phase HPLC employing a mobile phase composed of 7% water in methanol at a flow rate of 1.5 mL/min with UV detection at 254 nm. Radioactivity in column eluents was quantified by scintillation spectrometry (•).

global ischemia by electrospray ionization mass spectroscopy suggested that their accumulation was mediated through a fatty acyl chain elongation pathway. Fatty acyl chain elongation during ischemia was also suggested by the increased ratio of octadecanoyl carnitine mass to that of octadecadienoyl and octadecenoyl carnitine in ischemic myocardium perfused with supraphysiologic concentrations of hexadecanoic acid as compared to ischemic myocardium that was not supplemented with exogenous hexadecanoic acid. Proof of ischemia-induced fatty acyl chain elongation

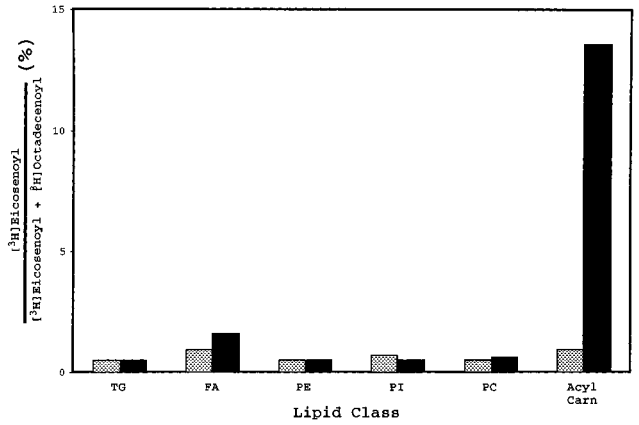

FIGURE 6: Incorporation of [9,10-^{3}H]octadec-9′-enoic acid into octadecenoic and eicosenoic residues of fatty acid, triglyceride, phospholipid, and acylcarnitine molecular species produced in control and globally-ischemic rabbit myocardium prelabeled with [9,10-^{3}H]octadec-9′-enoic acid. Rabbit hearts were prelabeled with [9,10-^{3}H]octadec-9′-enoic acid prior to control perfusion (shaded bars) or global ischemia for 20 min (solid bars). Myocardial triglycerides (TG), fatty acids (FA), phosphatidylethanolamines (PE), phosphatidylinositols (PI), phosphatidylcholines (PC), and acylcarnitines (Acyl Carn) were sequentially extracted from myocardium (1 g) and purified by HPLC, and endogenous aliphatic constituents were converted to their bromophenacyl ester derivatives prior to their separation as described in Experimental Procedures. Radioactivity in column eluents was quantified by scintillation spectrometry and was expressed as the percentage of radioactivity incorporated into eicosenoyl residues compared to radioactivity in both eicosenoyl and octadecenoyl residues in each lipid pool.

was further accrued by demonstrating the conversion of [9,-10-^{3}H]octadec-9′-enoic acid to [^{3}H]eicosenoyl carnitine in ischemic, but not control, myocardium. Accelerated conversion of [9,10-^{3}H]octadec-9′-enoic acid to eicosenoyl residues in the non-esterified fatty acid, triglyceride, and phospholipid pools was not manifest in ischemic myocardium, demonstrating the metabolic compartmentation of fatty acyl chain elongation during the ischemic process. The most likely explanation underlying these observations is that ischemia-induced fatty acyl chain elongation predominantly occurs in the mitochondrial compartment leading to the selective metabolic trapping of chain-elongated lipids as their carnitine derivatives (i.e., acylcarnitines). During ischemia, these acylcarnitines are blocked from further oxidative metabolism by NADH-mediated inhibition of fatty acid oxidation. In contrast, lipids synthesized in extramitochondrial compartments (e.g., triglycerides, phospholipids, etc.) do not contain chain-elongated products since the microsomal enzymes which catalyze their synthesis do not have access to non-esterified chain-elongated products.

Collectively, this study identifies unsaturated acylcarnitines as the predominant acylcarnitine molecular species accumulating in ischemic myocardium whose precise molecular composition is influenced by compartmentalized fatty acyl chain elongation. Since prior studies have demonstrated the selective accumulation of acylcarnitines in the sarcolemmal compartment of metabolically compromised myocytes (Wu et al., 1993), it seems likely that the novel acylcarnitine molecular species described herein may serve as important biochemical mediators of altered sarcolemmal membrane function during the ischemic process.

REFERENCES

Bligh, E. G., & Dyer, W. J. (1959) *Can. J. Biochem. Physiol. 37*, 911−917.
Cao, S. G., & Hatch, G. M. (1994) *Lipids 29*, 475−480.
Chien, K. R., Han, A., Sen, A., Buja, L. M., & Willerson, J. T. (1984) *Circ. Res. 54*, 313−322.
Christophersen, B. O., & Bremer, J. (1972) *Biochim. Biophys. Acta 260*, 515−526.
Chung, H. C., & Fleming, N. (1995) *Pflügers Arch. 429*, 789−796.
Corr, P. B., Snyder, D. W., Cain, M. E., Crafford, W. A., Jr., Gross, R. W., & Sobel, B. E. (1981) *Circ. Res. 49*, 354−363.
Corr, P. B., Gross, R. W., & Sobel, B. E. (1984) *Circ. Res. 55*, 135−154.
Durst, H. D., Milano, M., Kikta, E. J., Jr., Connelly, S. A., & Grushka, E. (1975) *Anal. Chem. 47*, 1797−1801.
Ford, D. A., & Gross, R. W. (1989) *Circ. Res. 64*, 173−177.
Gross, R. W. (1984) *Biochemistry 23*, 158−165.
Gross, R. W., & Sobel, B. C. (1982) *J. Biol. Chem. 258*, 5221−5226.
Hagve, T.-A., & Sprecher, H. (1989) *Biochim. Biophys. Acta 1001*, 338−344.
Han, X., & Gross, R. W. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 10635−10639.
Idell-Wenger, J. A., Grotyohann, L. W., & Neely, J. R. (1978) *J. Biol. Chem. 253*, 4310−4318.
Liedtke, A. J., & Nellis, S. H. (1979) *J. Clin. Invest. 64*, 440−447.
McGarry, J. D., & Foster, D. W. (1976) *J. Lipid Res. 17*, 277−281.
Mohammed, B. S., Hagve, T.-A., & Sprecher, H. (1990) *Lipids 25*, 854−858.
Okamura, S., & Yamashita, S. (1994) *J. Biol. Chem. 269*, 31207−31213.
Püttmann, M., Krug, H., von Ochsenstein, E., & Kattermann, R. (1993) *Clin. Chem. 39*, 825−832.
Sato, T., Arita, M., & Kiyosue, T. (1993) *Cardiovasc. Drugs Ther. 7*, 575−584.
Squire, R. S. T. (1991) *Anal. Biochem. 197*, 104−107.
Weishaar, R., Sarma, J. S. M., Maruyama, Y., Fischer, R., & Bing, R. J. (1977) *Cardiology 62*, 2−20.
Whitmer, J. T., Idell-Wenger, J. A., Rovetto, M. J., & Neely, J. R. (1978) *J. Biol. Chem. 253*, 4305−4309.
Wu, J., McHowat, J., Saffitz, J. E., Yamada, K. A., & Corr, P. B. (1993) *Circ. Res. 72*, 879−889.
Zierler, K. L. (1976) *Circ. Res. 38*, 459−463.

BI960552N