Differential Accumulation of Diacyl and Plasmalogenic Diglycerides During Myocardial Ischemia

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The recent discovery of neutral active choline and ethanamine glycerophospholipid specific phospholipase C in myocardium (Wolf RA, Gross RW. J Biol Chem 1985;260:7295) has demonstrated a novel catabolic pathway that potentially contributes to the accumulation of amphiphilic metabolites during myocardial ischemia. To assess the potential importance of this pathway, we quantified the temporal course of alterations in myocardial 1-0-alk-1'-enyl-2-acyl-sn-glycerol (AAG) and 1,2-diacyl-sn-glycerol (DAG) content during control and ischemic intervals in an isolated perfused Langendorf model. AAG accumulated over fivefold to 8.70 and 18.27 nmol/g dry in 20- and 60-minute ischemic rabbit hearts, respectively (p<0.02). The only AAG molecular species that was detected in substantial amounts in control or ischemic rabbit hearts was 1-0-hexadec-1'-enyl-2-acyl-sn-glycerol. Since this molecular species is enriched in plasmenylcholine these findings suggest that AAG production is likely mediated by phospholipase C-catalyzed hydrolysis of plasmenylcholine. In contrast to ischemia-induced AAG accumulation, DAG content decreased during both control and globally ischemic perfusion intervals. In summary, these findings demonstrate that AAG, in contrast to DAG, accumulates during myocardial ischemia indicating that at least some metabolites of plasmalogen and diacyl phospholipids accumulate at differential rates during myocardial ischemia. (Circulation Research 1989;64:173-177)
modified Krebs-Henseleit buffer. The perfusate was equilibrated with 95% \( O_2 \)-5% \( CO_2 \), the perfusion pressure was held constant at 60 mm Hg (average coronary flow of 25 ml/min) and hearts were paced at 180 beats/min. After an initial 10-minute perfusion period, hearts were either perfused for an additional 5, 20, or 60 minutes (control) or perfusion was terminated (zero-flow ischemia) while the hearts remained paced in a heating chamber for 5, 20, or 60 minutes. At the end of each interval, hearts were immediately freeze-clamped at liquid nitrogen temperature and myocardial wafers were pulverized into a fine powder with a stainless steel mortar and pestle. A 300–500 mg sample was taken for wet versus dry weight determination while the remainder of the sample was weighed prior to lipid extraction by the method of Bligh and Dyer. Appropriate internal standards were added during lipid extraction and myocardial lipids were quantified by high-performance liquid chromatography (HPLC) or capillary gas chromatography by comparisons with internal standards (1-0-eicosodec-9′-enyl-2-oleoyl-sn-glycerol, 1,2-diariaachidoyl-sn-glycerol, and arachidic acid).

Myocardial Lipid Analysis

Lipid constituents in the chloroform phase after Bligh and Dyer extraction were separated into neutral and polar lipid classes by silicic acid chromatography. 1-0-Alkyl-2-acyl-sn-glycerol and AAG were purified by thin layer chromatography (TLC) with silica gel G as stationary phase and a mobile phase comprised of petroleum ether/diethylether/acetic acid (70/30/1) as described above. Reduced with sodium bis (2-methoxyethoxy)-aluminum hydride (Vitride; Kodak Chemicals), and reaction products were purified by silicic acid chromatography and benzoylated with benzoic anhydride utilizing dimethylaminopyridine as catalyst. The resultant 1-0-alkyl-2,3-dibenzoyl-sn-glycerol and 1-0-alk-1′-enyl-2,3-dibenzoyl-sn-glycerol were resolved from fatty alcohol benzoates by TLC and were quantitated by reverse phase HPLC by comparisons with internal standards. Additional aliquots of the chloroform extract were used to purify DAG by HPLC utilizing an Ultrasphere-Si column (4.6×250 mm, 5 \( \mu \)m) as the stationary phase by isocratic elution with a mobile phase comprised of hexane/isopropanol/water (100/0.8/0.01 vol/vol/vol) at a flow rate of 2 ml/min. Column eluents were monitored by ultraviolet detection at 205 nm. In this system, DAG eluted from the column at approximately 10 minutes. Column eluents containing DAG were quantitated by capillary gas chromatographic analysis after acid-catalyzed methanolysis by comparisons with internal standards. Fatty acids were purified from the chloroform extract by TLC utilizing silica gel G plates (1 mm) with a mobile phase comprised of petroleum ether/diethylether/acetic acid (70/30/1) and were quantitated by capillary gas chromatographic analysis of their derivatized methyl esters by comparisons with internal standards.

The internal standard for fatty acid analysis, arachidic acid, was purchased from Nu Chek Prep (Elysian, Minnesota). However, the other internal standards utilized for AAG and DAG quantification, 1-0-eicosodec-9′-enyl-2-oleoyl-sn-glycerol and 1,2-diariaachidoyl-sn-glycerol, were not commercially available. The synthesis of 1-0-eicosodec-9′-enyl-2-oleoyl-sn-glycerol utilized dimethylaminopyridine catalyzed acylation of 1-0-eicosodec-9′-enyl-3-sn-glycerol (Foxboro Company, North Haven, Connecticut) by oleoyl chloride (Nu Chek Prep) with subsequent sn-3 specific decylation of 1-0-eicosodec-9′-enyl-2,3-dioleoyl-sn-glycerol catalyzed by Rhizopus arrhizus triglyceride lipase. Straight phase HPLC (as described above) was employed to purify 1-0-eicosodec-9′-enyl-2-oleoyl-sn-glycerol (retention time, 6.5 min) and 1,2-diariaachidoyl-sn-glycerol (retention time, 10.0 min) from the mixed isomers of diarachidin commercially available from Sigma Chemical, St. Louis, Missouri.

Results

Altered Myocardial Diglyceride Content During Global Ischemia

To delineate the effects of myocardial ischemia on the accumulation of myocardial neutral lipids potentially mediated by choline and ethanolamine glycerophospholipid specific phospholipase C, isolated Langendorf perfused rabbit hearts were rendered ischemic by cross-clamping the aortic inflow canula. Individual molecular species of AAG in control and ischemic hearts were quantified utilizing an internal standard (1-0-eicosodec-9′-enyl-2-oleoyl-sn-glycerol) by reverse phase HPLC as described in "Materials and Methods." Increases in the sixteen carbon vinyl ether molecular species of AAG, 1-0-hexadec-1′-enyl-2-acyl-sn-glycerol, were noted as rapidly as 5 minutes after ischemia (the first time point observed) and were maintained throughout the 60-minute experimental interval (Figure 1). In the 5-, 20-, and 60-minute zero-flow hearts, there were 28% (NS), 66% (p<0.01), and 84% (p<0.02) increases in 1-0-hexadec-1′-enyl-2-acyl-sn-glycerol in comparisons with controls. It is important to note that the levels of AAG in hearts subjected to 60 minutes of global ischemia contained over five times the mass present in either control or ischemic hearts after 5 minutes. No alterations between control and ischemic hearts in 1-0-hexadecyl-2-acyl-sn-glycerol content were noted. Other molecular species of AAG (e.g. 1-0-octadec-1′-enyl-2-acyl-sn-glycerol) were not detectable in substantial amounts in control or ischemic hearts.

A statistically significant time-dependent decrease in myocardial DAG content occurred during the perfusion interval in both control and ischemic hearts (Figure 2). Analysis of individual molecular species of DAG demonstrated that in both control-perfused and zero-flow ischemic hearts the predominant aliphatic constituents were palmitic, linoleic,
oleic, and stearic acids (Table 1). Since these fatty acid constituents are present in similar amounts in phosphatidylcholine but not phosphatidylinositol, these results suggest that the majority of DAG which is present during control or ischemic conditions originates from phospholipase C-mediated hydrolysis of phosphatidylcholine.3,5,6

To assess the temporal course of alterations in myocardial lipid metabolism during ischemia in this model, the accumulation of individual molecular species of fatty acid was quantitated. Since arachidonic acid is localized predominantly in endogenous phospholipid storage depots, accumulation of free arachidonic acid has gained widespread acceptance as a marker of accelerated myocardial phospholipid catabolism.14 During 20 minutes of myocardial ischemia a 16% increase in free fatty acid was present in comparison to control-perfused hearts (Table 2). After 60 minutes of global ischemia, a 315% increase in fatty acid content was manifest (p<0.005) (Table 2). This increase included a 15-fold increase in free arachidonic acid content while smaller fractional increases in other free fatty acids were also manifest. These results are qualitatively similar to both the mass of free fatty acids and the fractional increases in individual free fatty acids that others have previously demonstrated.14

Discussion

The present study documents the accumulation of a plasmalogen catabolite that increases over fivefold during 1 hour of myocardial ischemia. The data indicates that AAG increases and DAG decreases in both control and ischemic Langendorf perfused hearts which likely reflects the compromised biochemical integrity of buffer perfused hearts and underscores the lability of the AAG and DAG pools to pathophysiologic perturbations. Although unequivocal assignment of the molecular class responsible for the generation of 1-0-hexadec-1'-enyl-2-
acetyl-sn-glycerol cannot be determined from the present study consideration of the individual molecular species containing vinyl ether linkages in myocardium suggests that plasmenylcholine is the likely precursor for the majority of AAG which accumulates during the ischemic interval. Since plasmenylcholine contains the overwhelming majority of molecular species with 16 carbon vinyl ethers at the sn-1 position, the simplest, but not the only, explanation for the observed experimental data is that phospholipase C-catalyzed hydrolysis of plasmenylcholine is activated during myocardial ischemia resulting in the accumulation of AAG with 16 carbon aliphatic constituents at the sn-1 carbon.

It is important to note the discordance between the accumulation of diglyceride molecular species with vinyl ether linkages at the sn-1 position in comparison to the decline of DAG during both control and ischemic perfusion intervals. Prior studies have demonstrated that diglycerides are metabolized by the ordered sequential actions of diglyceride lipases that first cleave the sn-1 linked ester and subsequently cleave the sn-2 linked ester from the glycerol backbone. Since AAG is not susceptible to hydrolysis by conventional esterolytic lipases, we suspect that one factor contributing to the accumulation of AAG molecular species during ischemia is that they are metabolized less rapidly than their DAG counterparts. The possibility that these two diglyceride subclasses are phosphorylated at differential rates cannot be excluded but is difficult to assess, since phosphatidic acid extraction from tissues requires utilization of acidified extraction media, which results in hydrolysis of the vinyl ether bond.

It is instructive to compare the present results with those obtained by Chien et al. in a previous study examining alterations in myocardial DAG content in an in vivo canine model. Chien and coworkers also demonstrated substantial decreases as a function of time in DAG from both normal and ischemic myocardium that was comprised of similar molecular species as the DAG described herein. However, since myocardial choline and ethanolamine glycerophospholipid specific phospholipase C had not been discovered at the time that study was performed, and because of the considerable technical obstacles in the measurement of AAG mass, Chien et al did not quantify AAG mass as a marker of plasmenylcholine catabolism.

In summary, the present results demonstrate that AAG accumulates over fivefold during myocardial ischemia in an isolated perfused Langendorf model. Examination of the molecular species that are produced suggests that AAG accumulation results from phospholipase C-mediated hydrolysis of plasmenylcholine. The results also suggest that the majority of DAG present in myocardium during control and ischemic conditions does not originate from phospholipase C catalyzed hydrolysis of phosphatidylinositol. The biological significance of AAG accumulation during myocardial ischemia, either as a potential activator of specific isoforms of myocardial protein kinase C or as a modulator of sarcoplasmal molecular dynamics during ischemia, remains to be elucidated.

References

### Table 2. Fatty Acid Content in Control and Ischemic Rabbit Myocardium

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<th>Group</th>
<th>n</th>
<th>16:0</th>
<th>18:0</th>
<th>18:1</th>
<th>18:2</th>
<th>20:4</th>
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<td>4</td>
<td>88±19</td>
<td>28±11</td>
<td>33±5</td>
<td>35±6</td>
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<td>43±13</td>
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<td>2±1</td>
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<tr>
<td>60-minute ischemic</td>
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<td>213±27*</td>
<td>93±13*</td>
<td>118±17*</td>
<td>148±20*</td>
<td>29±8*</td>
<td>600±70*</td>
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</table>

Fatty acid content was analyzed from perfused (control) and zero-flow (ischemic) rabbit hearts as described in "Materials and Methods." Each value represents the mean of 5 determinations ±SEM and is given in nanomoles per gram dry weight. *p<0.005 for comparisons between control and ischemic hearts at 60 minutes.


Key Words • myocardial ischemia • diglycerides • plasmalogens
American Heart Association

Scientific Sessions

1989

New Orleans, Louisiana
November 13-16

1990

Dallas, Texas
November 12-15

1991

Anaheim, California
November 18-21

1992

New Orleans, Louisiana
November 9-12
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Circ Res. 1989;64:173-177
doi: 10.1161/01.RES.64.1.173

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/64/1/173

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