Differential Accumulation of Diacyl and Plasmalogenic Diglycerides During Myocardial Ischemia

David A. Ford and Richard W. Gross

The recent discovery of neutral active choline and ethanolamine 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)

During the last decade many studies have demonstrated profound alterations in phospholipid metabolism during myocardial ischemia and have implicated these alterations as important biochemical determinants of electrophysiologic dysfunction and myocytic cell death.1-4 Recently, plasmalogen molecular species have been identified as the predominant phospholipid constituents of canine myocardial sarcolemma3 and sarcoplasmic reticulum.6 Despite the numerous studies on alterations in myocardial phospholipid metabolism during myocardial ischemia, changes in the catabolism of the predominant phospholipid class of sarcolemma and sarcoplasmic reticulum (i.e., plasmenylcholine and plasmeneylethanolamine) during ischemia have been virtually ignored.

We have identified a novel phospholipase C in myocardium which hydrolyzes ethanolamine and choline glycerophospholipids including plasmalogen molecular species.7 Subsequently, a variety of groups have demonstrated accumulation of 1,2-diacyl-sn-glycerol (DAG) in liver, brain, and MDCK cells during agonist stimulation which was comprised predominantly of 1-palmitoyl-2-oleoyl molecular species, implicating the importance of phospholipase C-mediated hydrolysis of phosphatidylcholine in signal transduction processes.8-10 Accordingly, the present study was performed to assess the accumulation of 1,0-alk-1'-enyl-2-acyl-sn-glycerol (AAG) during myocardial ischemia which reflects, at least in part, hydrolysis of plasmenylcholine and plasmeneylethanolamine, which are highly enriched in the sarcolemmal and sarcoplasmic reticular compartments. The results demonstrate substantial increases in AAG content during global ischemia in Langendorf perfused rabbit hearts while DAG content decreased monotonically during this interval.

Materials and Methods

Langendorf Perfusion of Rabbit Myocardium

Rabbits were anesthetized under diethylether and hearts were excised and perfused retrograde with
modified Krebs-Henseleit buffer. The perfusate was equilibrated with 95% O₂-5% CO₂, 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, the 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-di-arachidoyl-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) (Rf=0.38), reduced with sodium benzoates by TLC and were quantitated by reverse phase HPLC utilizing a stainless steel column (4.6x250 mm, 5 μm) as stationary phase and 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 (Elkton, Minnesota). However, the other internal standards utilized for AAG and DAG quantification, 1-0-eicosodec-9'-enyl-2-oleoyl-sn-glycerol and 1,2-di-arachidoyl-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-sn-glycerol (Foxboro Company, North Haven, Connecticut) by oleoyl chloride (Nu Chek Prep) with subsequent sn-3 specific deacylation 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-di-arachidoyl-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-hexadec-1'-enyl-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.\(^1\)\(^4\) 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.\(^1\)\(^4\)

**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-
vinyl ether bond.

extraction media, which results in hydrolysis of the
tination from tissues requires utilization of acidified
difficult to assess, since phosphatidic acid extrac-
ted at differential rates cannot be excluded but is
these two diglyceride subclasses are phosphory-
ized by the ordered sequential actions of diglyc-
eride lipases that first cleave the

sn-1

olized with vinyl ether linkages at the

sn-1

carbon aliphatic constituents at the

sn-2

position in

sn-1

with vinyl ether linkages at the

sn-1

carbon position in

sn-1

position 56 the simplest, but not the only,
explanation for the observed experimental data is
that phospholipase C-catalyzed hydrolysis of plas-
menylcholine 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 metabo-
lized by the ordered sequential actions of diglyc-
eride 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 suscepti-
tible 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 phosphory-
lated at differential rates cannot be excluded but is
difficult to assess, since phosphatidic acid extrac-
tion 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 ethanol-
amine 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 pro-
duced suggests that AAG accumulation results from
phospholipase C-mediated hydrolysis of plasmenyl-
choline. The results also suggest that the majority of
DAG present in myocardium during control and
ischemic conditions does not originate from phos-
pholipase C catalyzed hydrolysis of phosphatidyl-
inositol. The biological significance of AAG accumu-
lation during myocardial ischemia, either as a
potential activator of specific isofoms of myocardial
protein kinase C or as a modulator of sarcolem-
mal molecular dynamics during ischemia, remains
to be elucidated.

References

<table>
<thead>
<tr>
<th>Group</th>
<th>16:0</th>
<th>18:0</th>
<th>18:1</th>
<th>18:2</th>
<th>20:4</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-minute control</td>
<td>27</td>
<td>20</td>
<td>15</td>
<td>29</td>
<td>9</td>
<td>1.43±0.14</td>
</tr>
<tr>
<td>5-minute ischemic</td>
<td>28</td>
<td>18</td>
<td>16</td>
<td>30</td>
<td>5</td>
<td>1.77±0.13</td>
</tr>
<tr>
<td>20-minute control</td>
<td>28</td>
<td>18</td>
<td>18</td>
<td>30</td>
<td>6</td>
<td>1.09±0.13</td>
</tr>
<tr>
<td>20-minute ischemic</td>
<td>30</td>
<td>15</td>
<td>19</td>
<td>30</td>
<td>5</td>
<td>1.53±0.11</td>
</tr>
<tr>
<td>60-minute control</td>
<td>31</td>
<td>16</td>
<td>17</td>
<td>27</td>
<td>8</td>
<td>0.47±0.09</td>
</tr>
<tr>
<td>60-minute ischemic</td>
<td>37</td>
<td>11</td>
<td>20</td>
<td>31</td>
<td>2</td>
<td>1.05±0.04</td>
</tr>
</tbody>
</table>

1,2-Diacyl-sn-glycerol content was analyzed from perfused (control) and zero-flow (ischemic) rabbit hearts as
described in "Materials and Methods." Each value represents the mean of five determinations ±SEM. Total values
of 1,2-diacyl-sn-glycerol are given in micromoles per gram dry weight and values for individual fatty acid moieties
are expressed as percentages of the total fatty acid in 1,2-diacyl-sn-glycerol.

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

<table>
<thead>
<tr>
<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>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>20-minute control</td>
<td>4</td>
<td>88±19</td>
<td>28±11</td>
<td>33±5</td>
<td>35±6</td>
<td>2±1</td>
<td>190±40</td>
</tr>
<tr>
<td>20-minute ischemic</td>
<td>6</td>
<td>93±19</td>
<td>43±13</td>
<td>43±8</td>
<td>40±6</td>
<td>3±1</td>
<td>220±40</td>
</tr>
<tr>
<td>60-minute control</td>
<td>4</td>
<td>83±11</td>
<td>20±8</td>
<td>40±6</td>
<td>40±4</td>
<td>2±1</td>
<td>190±20</td>
</tr>
<tr>
<td>60-minute ischemic</td>
<td>4</td>
<td>213±27*</td>
<td>93±13*</td>
<td>118±17*</td>
<td>148±20*</td>
<td>29±8*</td>
<td>600±70*</td>
</tr>
</tbody>
</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 n determinations ±SEM and is given in nanomoles
per gram dry weight. *p<0.005 for comparisons between control and ischemic hearts at 60 minutes.
in canine purkinje fibers induced by lysophosphoglycerides. 

1. Katz AM, Messineo FC: Lipid-membrane interactions and the pathogenesis of ischemic damage in the myocardium. 

2. Shaikh NA, Downar E: Time course of changes in porcine myocardial phospholipid levels during ischemia: A reassessment of the lysolipid hypothesis. 


4. Bocckino SB, Blackmore PF, Exton JH: Stimulation of 1,2-diacylglycerol accumulation in hepatocytes by vasopressin, epinephrine, and angiotensin II. 

5. Bligh EG, Dyer WJ: A rapid method of total lipid extraction and purification. 

6. Wolf RA, Gross RW: Identification of neutral active phospholipase C which hydrolyzes choline glycerophospholipids and plasmalogen selective phospholipase A2 in canine myocardium. 


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
Differential accumulation of diacyl and plasmalogenic diglycerides during myocardial ischemia.

D A Ford and R W Gross

_Circ Res._ 1989;64:173-177
doi: 10.1161/01.RES.64.1.173

_Circulation Research_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 1989 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

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

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in _Circulation Research_ can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to _Circulation Research_ is online at:
http://circres.ahajournals.org/subscriptions/