Reactive Oxygen Species–Induced Stimulation of 5′AMP-Activated Protein Kinase Mediates Sevoflurane-Induced Cardioprotection

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Background—5′AMP-activated protein kinase (AMPK), a well-known regulator of cellular energy status, is also implicated in ischemic preconditioning leading to cardioprotection. We hypothesized that AMPK is involved in anesthetic-induced cardioprotection and that this activation is mediated by reactive oxygen species (ROS).

Methods and Results—Isolated Langendorff-perfused rat hearts were subjected to 35 minutes of global ischemia (I) followed by 120 minutes of reperfusion (I/R). Hearts were assigned to a control group (Con) or a sevoflurane (Sevo) group receiving 3 times 5-minute episodes of sevoflurane (2.5vol%) before I/R. Phosphorylation of both AMPK and endothelial nitric oxide synthase (eNOS) were determined by Western blot analysis. Cardioprotection was assessed after I/R from recovery of left ventricular pressure and from infarct size (triphenyltetrazolium chloride staining). In the control group, ischemia resulted in a 2-fold increase in phosphorylation levels of AMPK (Con 0.13 ± 0.01 versus Con-I 0.28 ± 0.05, $P<0.05$), which was sustained after 120 minutes of reperfusion (Con-I/R 0.26 ± 0.02, $P<0.05$). Sevoflurane preconditioning had no affect on AMPK phosphorylation before ischemia (Sevo 0.12 ± 0.03, $P>0.05$), but almost doubled the increase in AMPK phosphorylation relative to control after ischemia (Sevo-I 0.48 ± 0.09, $P<0.05$), an effect that was sustained after reperfusion (Sevo-I/R 0.49 ± 0.12, $P<0.05$). The AMPK-inhibitor compound C (10 μmol/L) reduced the sevoflurane-mediated increase in phosphorylation of AMPK and its target eNOS and abolished cardioprotection. The ROS-scavenger n-(2-mercaptopropionyl)-glycine (1 mmol/L) blunted the sevoflurane-mediated increase in AMPK and eNOS phosphorylation and prevented cardioprotection.

Conclusions—Sevoflurane-induced AMPK activation protects the heart against ischemia and reperfusion injury and relies on upstream production of ROS. (***Circulation*. 2009;120[suppl 1]:S10–S15.)**

Key Words: ischemia ■ signal transduction ■ free radicals ■ volatile anesthetics ■ protein kinase

The heart possesses intrinsic protective mechanisms against ischemia and reperfusion (I/R) injury, which are induced by brief ischemic episodes and rely on protein kinase C (PKC), glycogen synthase kinase 3β (GSK3β), and on production of reactive oxygen species (ROS).[1] Volatile anesthetics, such as sevoflurane, exert cardioprotective properties as well and operate via similar signaling cascades.[2–4] For instance, sevoflurane-induced production of ROS during the preconditioning period is involved in cardioprotection,[3] whereas sevoflurane preconditioning also reduces the excessive production of ROS after I/R.[5] The in vivo relevance of these processes is demonstrated by De Hert et al, who showed that sevoflurane improved cardiac function in patients during coronary artery bypass surgery[6] and in high-risk patients with reduced cardiac function.[7]

5′AMP-activated protein kinase (AMPK), an important sensor and regulator of cellular energy status, is activated in response to ischemic stress and is implicated in ischemic preconditioning.[8] In nonischemic hearts, AMPK is activated by hydrogen peroxide (H_2O_2)[9] and peroxynitrite ($ONOO^-$).[10] Currently, it is unknown whether AMPK plays a role in volatile anesthetics-induced cardioprotection.

Accordingly, we hypothesize that AMPK activation is involved in anesthetic-induced cardioprotective signaling and that this activation requires production of ROS. Elucidation of the involvement of AMPK in anesthetic-induced cardioprotective signaling may have important implications for the development of clinical cardioprotective strategies, in particular during diabetes mellitus, where intrinsic AMPK-dependent signaling is depressed.[8]

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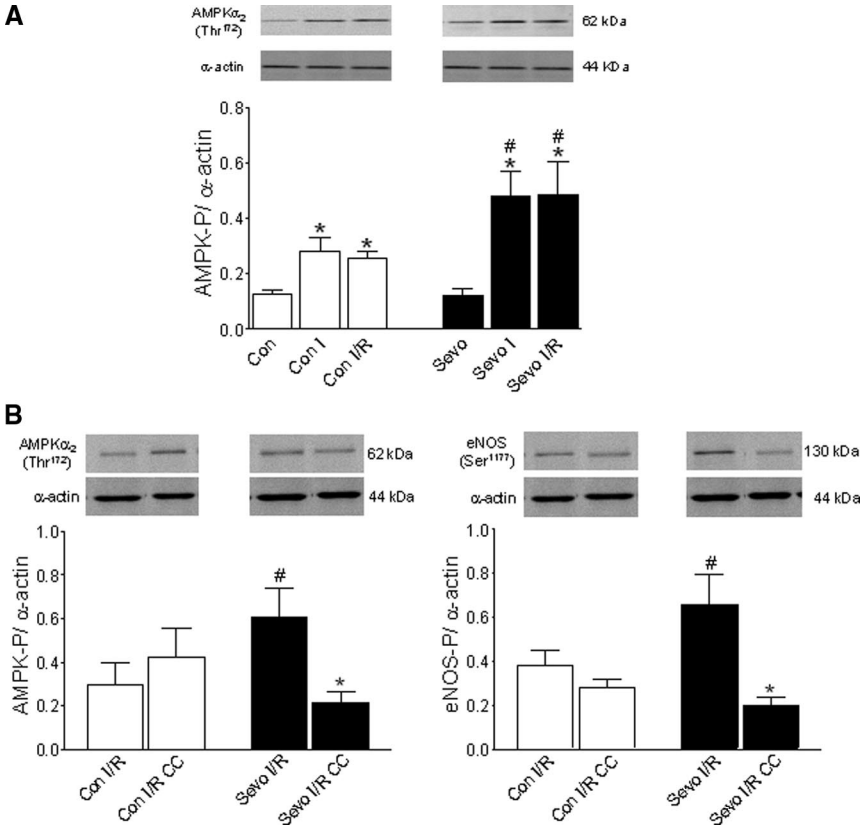

Figure 1. Sevoflurane enhances the ischemia-induced increase in AMPK phosphorylation, which is inhibited by the AMPK-inhibitor compound C. **A**, Examples and averaged values of phosphorylation levels of AMPKα at Thr^{172} in left ventricular tissue in the control (Con) and sevoflurane (Sevo) group before ischemia, after 35 minutes of ischemia (I), and after ischemia and 120 minutes of reperfusion (I/R). The increase in AMPK-phosphorylation after I/R and I were enhanced in the Sevo group compared to control group. Values are expressed as means±SEM, n=6 per group, #$P<0.05$ Sevo vs Control, *$P<0.05$ vs preischemia (post hoc). **B**, Examples and averaged values of the phosphorylation levels of AMPK, and eNOS at Ser^{1177}, in the absence and presence of AMPK-inhibitor compound C (CC, 10 μmol/L). The 2-way ANOVA (interaction) revealed that the effect of CC in the control and sevoflurane group differed both for AMPK and eNOS phosphorylation. The sevoflurane-induced increase in AMPK and eNOS phosphorylation after I/R was normalized to control values by CC. Values are expressed as means±SEM, n=6 per group, #$P<0.05$ Sevo vs Control, *$P<0.05$ CC vs non-CC (post hoc).

To test our hypothesis we determined, in isolated Langendorff-perfused rat hearts, the effect of sevoflurane on phosphorylation of AMPK before ischemia, after ischemia, and after reperfusion, as well as myocardial recovery and infarct size after reperfusion. The functional contribution of AMPK was assessed with a specific AMPK-inhibitor, and a ROS-scavenger was used to assess the involvement of ROS.

Methods

An expanded version of Methods is provided in the Online Data Supplement.

Animals and Experimental Set-Up

This study was performed in accordance with the guidelines of the Institutional Animal Care and Use Committee of the VU University Medical Center (VUMC), which conforms to the *Guide for the Care and Use of Laboratory Animals* (NIH Publication No. 85-23, revised 1996). Male Wistar rats (n=116, 250 to 400g; Harlan, The Netherlands) were anesthetized with pentobarbital (80 mg/kg, intraperitoneal). Subsequently, the heart was rapidly excised and mounted in a Langendorff set-up. Left ventricular (LV) pressures were measured with a balloon inserted in the LV to determine functional recovery.

Experimental Protocol and Assays

To determine whether sevoflurane induces AMPK phosphorylation, hearts were assigned to 2 groups: (1) Control (Con) group: no preconditioning; (2) Sevoflurane (Sevo) group: preconditioning by application of 3 times 5-minute episodes of sevoflurane (2.5vol%) separated by 5 minutes of washout. Hearts from both groups were frozen in liquid nitrogen immediately before ischemia, immediately after 35 minutes of global ischemia (I), or after 35 minutes of global ischemia followed by 120 minutes of reperfusion (I/R).

The contribution of sevoflurane-induced activation of AMPK and the involvement of ROS to the improved myocardial recovery and reduced infarct size were investigated by subsequently assigning hearts of the control and sevoflurane group to subgroups identified by the following inhibitors: (1) reversible AMPK inhibitor compound C (CC, 10 and 1 μmol/L), (2) ROS-scavenger n-(2-mercaptopropionyl)-glycine (MPG, 1 mmol/L), (3) both MPG (1 mmol/L) and CC (10 μmol/L), and (4) NOS-inhibitor Nω-nitro-L-arginine (L-NA, 0.1 mmol/L).

Recovery of LV pressures was determined, and infarct size was assessed from triphenyltetrazolium chloride (TTC)-stained sections. Western blot analysis of LV apex samples was used for determination of AMPK phosphorylation with an antibody against phospho-AMPKα at Thr^{172}. AMPK activity was determined by measuring eNOS phosphorylation with an antibody against phospho-eNOS at Ser^{1177}.

Statistical Analysis

Levels of AMPK phosphorylation between the control and sevoflurane group at 3 different time points (before ischemia, after ischemia, after I/R) were obtained in different hearts and therefore analyzed using 2-way ANOVA. Differences in AMPK and eNOS phosphorylation, recovery of cardiac function ($P_{developed}$, $P_{diastolic}$, and +dP/dt) and infarct size between the control and sevoflurane groups with and without CC were also analyzed with 2-way ANOVA, whereas the effects of MPG and the combination of MPG+CC were analyzed with multiple ANOVA. The interaction term indicated whether the effect of the inhibitor was different between the control and sevoflurane group. A Bonferroni post hoc test was used to assess the effect of the inhibitor within the control and sevoflurane group. A value of $P<0.05$ was considered significant. All data are expressed as means±SEM.

Statement of Responsibility

The authors had full access to the data and take responsibility for its integrity. All authors have read and agree to the manuscript as written.

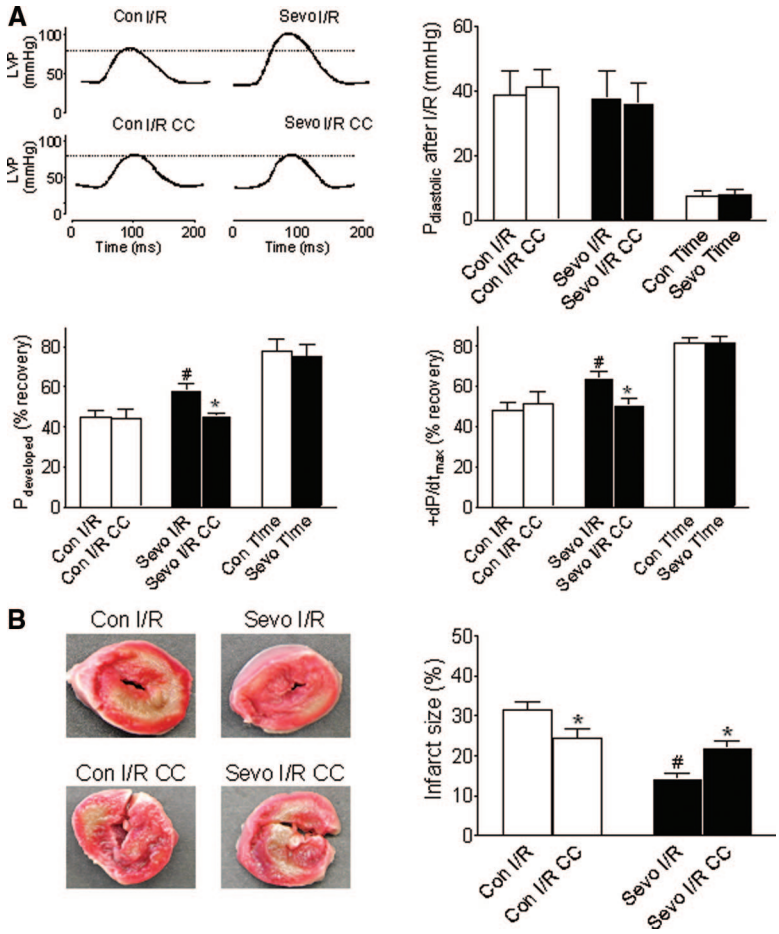

Figure 2. Contribution of sevoflurane-induced AMPK activation to cardioprotection **A**, Examples of left ventricular pressure (LVP) tracing during a contraction and averaged data of LV $P_{diastolic}$, recovery of $P_{developed}$ and recovery of $+dP/dt_{max}$ in isolated rat hearts in the control (Con) and sevoflurane (Sevo) group after ischemia and reperfusion (I/R) in the presence of AMPK-inhibitor compound C (CC, 10 μmol/L) and in time control groups. **B**, Examples and averaged data of infarct size determined with TTC staining in isolated rat hearts in control and sevoflurane groups after I/R in the presence of CC. Two-way ANOVA revealed that preconditioning with 2.5vol% sevoflurane resulted in a significant increased recovery of $P_{developed}$ and a reduced infarct size compared to controls. The interaction in the 2-way ANOVA revealed that this effect was abolished by CC. Values are expressed as means±SEM, n=8 per group, #$P<0.05$ Sevo vs Control, *$P<0.05$ CC vs no-CC (post hoc).

Results

Sevoflurane Enhances the Ischemia-Induced Increase in AMPK-Phosphorylation

In Figure 1A are shown representative examples and averaged values of the phosphorylation levels of AMPKα at Thr^{172} in LV cardiac tissue of the control and sevoflurane group, before ischemia, after ischemia, and after ischemia and reperfusion. In the control group, 35 minutes of ischemia resulted in an almost 2-fold increase in AMPK phosphorylation, an effect that was sustained after reperfusion. Before ischemia, sevoflurane preconditioning had no effect on phosphorylation levels of AMPK. However, after ischemia, sevoflurane preconditioning resulted in an almost 4-fold increase in AMPK-phosphorylation relative to the control group before ischemia, which was sustained after reperfusion. This implies a 2-fold increase of AMPK phosphorylation by sevoflurane preconditioning during ischemia.

AMPK Inhibition Blocks Sevoflurane-Induced Increase in AMPK Phosphorylation and Activity

Figure 1B shows that the sevoflurane-induced increase in AMPK phosphorylation after I/R was blocked by addition of 10 μmol/L of the AMPK-inhibitor compound C (CC), whereas in the Con I/R group, the levels of phosphorylation of AMPK remained unaffected, although a trend to increased levels existed. Addition of a lower dose (1 μmol/L) of CC had a similar effect (data not shown). The phosphorylation of eNOS, a well-known target of AMPK and an index of its activity, was increased in the Sevo I/R group compared to control I/R group. The AMPK-inhibitor CC (10 μmol/L) abolished the sevoflurane-induced increase in eNOS phosphorylation after I/R. This indicates that AMPK-phosphorylation and its activity were blocked. In addition, the NOS inhibitor L-NA (0.1 mmol/L) also abolished the sevoflurane-induced increase in eNOS phosphorylation after I/R (Con I/R L-NA: 0.22±0.04% versus Sevo I/R L-NA: 0.28±0.05%, n=5).

Sevoflurane-Induced AMPK Activation Contributes to Cardioprotection

Anesthetic preconditioning with sevoflurane was evident after I/R from an increased recovery of $P_{developed}$ and $+dP/dt_{max}$ compared to controls (Figure 2A, n=8). $P_{diastolic}$ values were not different between groups after I/R. This sevoflurane-induced improved functional cardiac recovery was abolished by addition of 10 μmol/L of the AMPK inhibitor CC (Figure 2A, n=8). A lower concentration of CC (1 μmol/L) also blocked the sevoflurane-induced improved functional recovery of $P_{developed}$ (Con I/R CC: 43±3% versus Sevo I/R CC: 47±5%, n=5). In addition, the NOS inhibitor L-NA also blocked the sevoflurane-induced improved functional recovery (Con I/R L-NA: 37.4±4% versus Sevo I/R L-NA: 43.1±5%, n=5). In time control experiments, without I/R, the LV $P_{developed}$ at the end of the experiment (200 minutes) amounted to approximately ≈80% of the value before ischemia (Figure 2A).

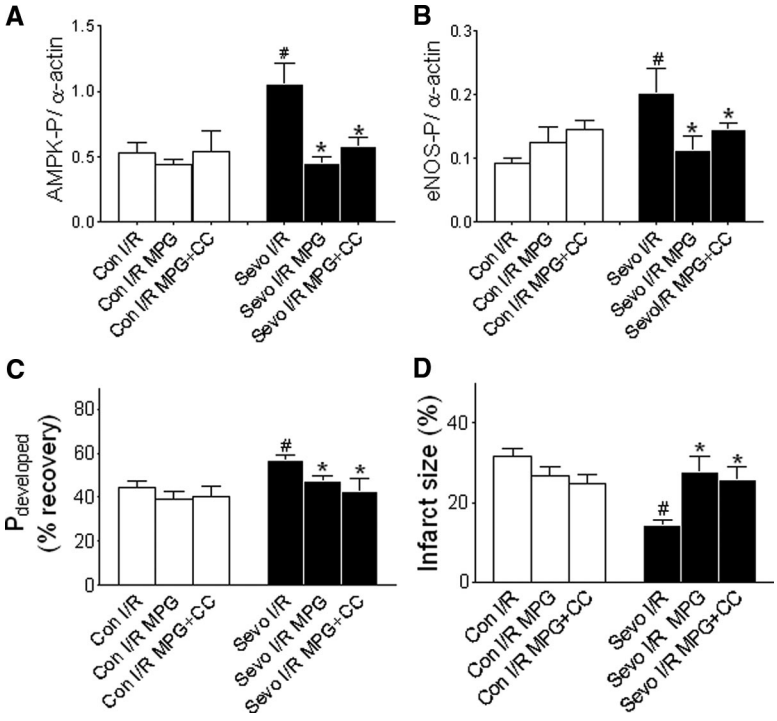

Figure 3. Sevoflurane-induced AMPK activation relies on production of ROS. Phosphorylation of AMPK (A), phosphorylation of eNOS (B), myocardial function (C), and infarct size (D) in control (Con) and sevoflurane (Sevo) groups after ischemia and reperfusion (I/R) in the presence of ROS-scavenger MPG (1 mmol/L) and in the presence of MPG and AMPK inhibitor compound C (CC, 10 μmol/L). Multiple ANOVA revealed that MPG reduced the sevoflurane-induced increase in AMPK and eNOS phosphorylation, and abolished sevoflurane-induced protective effects on myocardial function and infarct size. No additional effects of CC on the effects of MPG were found. Values are expressed as means±SEM, n=5 per group, #$P<0.05$ Sevo vs Control, *$P<0.05$ MPG (+CC) vs non-MPG (-CC).

The analysis of the TTC-stained heart slices shown in Figure 2B revealed that sevoflurane preconditioning substantially reduced infarct size after I/R compared to controls (n=8). The sevoflurane-induced reduction in infarct size was blocked by AMPK inhibitor CC at the high (10 μmol/L; Figure 2B, n=8), as well as the lower (1 μmol/L) concentration (Infarct size: Con I/R CC: 23±4% versus Sevo I/R CC: 22±3%, n=5). In control hearts CC decreased infarct size (Figure 2B). In addition, the NOS inhibitor L-NA (0.1 mmol/L) also blocked the sevoflurane-induced reduction in infarct size (Con I/R L-NA 28±4% versus Sevo I/R L-NA 26±4%, n=5).

In the time control group, without I/R, myocardial damage was only 1.6±0.8% (n=8). In additional experiments (n=3), we found that DMSO, used as vehicle for dissolving CC, did not affect the functional recovery after I/R ($P_{developed}$: 44±6%), nor did it reduce infarct size (30±4%).

Sevoflurane-Induced AMPK Activation Relies on Production of ROS

Scavenging of ROS by addition of 1 mmol/L MPG during sevoflurane preconditioning (n=5) reduced the phosphorylation levels of AMPK (Figure 3A) and eNOS (Figure 3B) after I/R. This was accompanied by blunting of functional recovery (Figure 3C) and an increased infarct size (Figure 3D). In the control group, without sevoflurane, no effects of MPG on AMPK or eNOS phosphorylation, functional cardiac recovery, and infarct size were observed (n=5). In addition, the combination of ROS scavenging (MPG) with AMPK inhibition (CC) resulted in phosphorylation levels of AMPK and eNOS (Figure 3A and 3B) that were not different between control and sevoflurane groups (both n=5) and that were equivalent to the levels found with either inhibitor alone. Similar results were found for functional recovery (Figure 3C) and the infarct size (Figure 3D).

Discussion

The main finding of this study is that sevoflurane pretreatment enhances AMPK activation during ischemia. This activation results in improved myocardial recovery and reduced infarct size, as became apparent from the experiments in which the AMPK-inhibitor compound C was used. In addition, ROS scavenging during sevoflurane preconditioning blunted AMPK activation and abolished cardioprotection. This indicates that sevoflurane-induced cardioprotection is mediated via ROS-dependent activation of AMPK. Our study demonstrates for the first time a direct link between AMPK activation and the production of ROS in anesthetic-induced cardioprotection.

The present study clearly shows that sevoflurane preconditioning enhances the increase in AMPK phosphorylation after ischemia, as well as after reperfusion. Although sevoflurane did not immediately activate AMPK (Figure 1A), it apparently primed the myocardium and doubled the increase in AMPK phosphorylation observed after ischemia, which persisted after reperfusion. This suggests that the ischemic conditions are necessary to activate AMPK. Phosphorylation of eNOS (Figure 1B), a well-known target of AMPK in cardioprotective signaling,[11] was increased as well, indicating that changes in AMPK phosphorylation reflect changes in AMPK activity. The sevoflurane-induced increase in AMPK and eNOS phosphorylation was blunted by the AMPK-inhibitor compound C. Compound C, as well as NOS inhibitor L-NA, abolished the effects of sevoflurane on myocardial recovery and infarct size, indicating that the sevoflurane-induced increased in phosphorylation of AMPK and eNOS during ischemia are functionally related to the cardioprotection.

In our control hearts compound C decreased infarct size, which was accompanied by a trend to increased levels of AMPK phosphorylation. Khaliulin et al[12] noted in isolated rat hearts that compound C enhanced cardioprotection induced by temperature preconditioning and suggested that this paradoxical effect of compound C might be related to altering the balance between the beneficial and detrimental AMPK activation during I/R.[13] The ischemia-induced AMPK activation has the potential to increase myocardial energy supply by glycolysis during ischemia and by glucose uptake during reperfusion, thereby exerting a cardioprotective effect. However, AMPK activation can also stimulate fatty acid oxidation, which can increase proton production and decrease cardiac efficiency by concomitant inhibition of glucose oxidation.[13] These dual effects of AMPK on metabolic pathways, ion channels, protein synthesis, and cellular function reflect its complex role during I/R. Our results show that inhibition of ROS with MPG completely abolished the sevoflurane-induced AMPK phosphorylation as well as cardioprotection. Hence, our results indicate that the additional activation of AMPK by sevoflurane during ischemia is beneficial as it protects against I/R injury. The absence of an effect of MPG on AMPK phosphorylation and infarct size in control hearts illustrates the complex role of AMPK during I/R-injury.

How does sevoflurane enhance AMPK activation during ischemia? During severe ischemia, intracellular ATP levels drop, resulting in a substantial rise of the intracellular concentration of 5′-AMP in the heart, which will lead to AMPK activation.[14] Sevoflurane does not influence intracellular 5′-AMP levels in the heart, and no effects of sevoflurane were found on intracellular myocardial ATP levels.[15] Thus, alternative mechanisms of AMPK activation should be considered.

The present study shows that the ROS-scavenger MPG blunted the sevoflurane-induced enhanced AMPK activation after I/R and that this was accompanied by an inhibition of cardioprotection. MPG was applied during the preconditioning period, thus before the ischemic period when AMPK activation took place. This indicates that sevoflurane-induced AMPK activation is ROS-dependent. In addition, combining ROS scavenging with AMPK inhibition did not result in an additional effect on AMPK activation or cardioprotection, indicating that the production of ROS is upstream of AMPK activation in the same signaling pathway. MPG scavenges hydroxyl radicals and $ONOO^-$ but not H_2O_2 and superoxide.[16] Moreover, $ONOO^-$ reduced myocardial infarct size in cats after I/R.[17] These data, in combination with previous observations from our laboratory showing that MPG is able to inhibit the sevoflurane-induced $ONOO^-$ modification of tyrosine residues,[3] indicate that $ONOO^-$ is the most likely mediator for sevoflurane-induced ROS-dependent activation of AMPK. The excessive ROS production during reperfusion is detrimental for the heart,[18] whereas small amounts of ROS generated during preconditioning prove to be beneficial,[5] as also indicated by the present study.

Several other upstream factors, such as the tumor suppressor protein LKB1, the calcium/calmodulin-dependent kinase kinase (CaMKK)-β,[19] or the kinase H11K,[20] are able to activate AMPK. Cardiac AMPK activation was also linked to activation of PKC and inhibition of GSK3β.[20,21] Volatile anesthetics, such as sevoflurane, isoflurane, and desflurane, also influence these kinases.[2–4,22–24] For instance, activation of different isoforms of PKC, which was preceded by ROS production, and extracellular signal-regulated kinase 1/2 (ERK1/2) have been demonstrated in volatile anesthetic-induced cardioprotective signaling.[3,22,23] In addition, isoflurane pre- and postconditioning was linked to activation of phosphatidylinositol 3-kinase inhibitor (PI3K)[24] and GSK3β.[4] Our experiments clearly indicate that sevoflurane-preconditioning alters ROS production, but future experiments will be needed to elucidate how these kinases relate to AMPK-dependent cardioprotective signaling.

The isolated Langendorff perfused heart preparation in present study was chosen to examine the underlying cardiac mechanism of sevoflurane cardioprotection under controlled ex vivo conditions to exclude possible confounding systemic hemodynamics and humoral effects of sevoflurane and the inhibitors. Caution should be exerted when extrapolating these findings to the in vivo situation, although the intracellular mechanism revealed most likely is important for anesthetic preconditioning in patients.

Conclusion

Sevoflurane-induced AMPK activation protects the heart against ischemia and reperfusion injury and relies on upstream production of ROS. Therefore, the present study indicates that AMPK is not only a key regulator of energy status, but also plays a pivotal role in anesthetic cardioprotection.

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Disclosures

None.

References

1. Hausenloy DJ, Yellon DM. Survival kinases in ischemic preconditioning and postconditioning. *Cardiovasc Res*. 2006;70:240–253.
2. Zaugg M, Lucchinetti E, Uecker M, Pasch T, Schaub MC. Anaesthetics and cardiac preconditioning. Part I Signalling and cytoprotective mechanisms. *Br J Anaesth*. 2003;91:551–565.
3. Bouwman RA, Musters RJ, Beek-Harmsen BJ, de Lange JJ, Boer C. Reactive oxygen species precede protein kinase C-delta activation independent of adenosine triphosphate-sensitive mitochondrial channel opening in sevoflurane-induced cardioprotection. *Anesthesiology*. 2004; 100:506–514.
4. Feng J, Lucchinetti E, Ahuja P, Pasch T, Perriard JC, Zaugg M. Isoflurane postconditioning prevents opening of the mitochondrial permeability transition pore through inhibition of glycogen synthase kinase 3beta. *Anesthesiology*. 2005;103:987–995.
5. Novalija E, Varadarajan SG, Camara AK, An J, Chen Q, Riess ML, Hogg N, Stowe DF. Anesthetic preconditioning: triggering role of reactive oxygen and nitrogen species in isolated hearts. *Am J Physiol Heart Circ Physiol*. 2002;283:H44–H52.
6. De Hert SG, ten Broecke PW, Mertens E, Van Sommeren EW, De Blier IG, Stockman BA, Rodrigus IE. Sevoflurane but not propofol preserves myocardial function in coronary surgery patients. *Anesthesiology*. 2002; 97:42–49.
7. De Hert SG, Cromheecke S, ten Broecke PW, Mertens E, De Blier IG, Stockman BA, Rodrigus IE, van der Linden PJ. Effects of propofol, desflurane, and sevoflurane on recovery of myocardial function after

coronary surgery in elderly high-risk patients. *Anesthesiology*. 2003;99: 314–323.
8. Young LH. AM. P-activated protein kinase conducts the ischemic stress response orchestra. *Circulation*. 2008;117:832–840.
9. Leon H, Atkinson LL, Sawicka J, Strynadka K, Lopaschuk GD, Schulz R. Pyruvate prevents cardiac dysfunction and AMP-activated protein kinase activation by hydrogen peroxide in isolated rat hearts. *Can J Physiol Pharmacol*. 2004;82:409–416.
10. Zou MH, Kirkpatrick SS, Davis BJ, Nelson JS, Wiles WG, Schlattner U, Neumann D, Brownlee M, Freeman MB, Goldman MH. Activation of the AMP-activated protein kinase by the anti-diabetic drug metformin in vivo. Role of mitochondrial reactive nitrogen species. *J Biol Chem*. 2004;279:43940–43951.
11. Chen ZP, Mitchelhill KI, Michell BJ, Stapleton D, Rodriguez-Crespo I, Witters LA, Power DA, Ortiz de Montellano PR, Kemp BE. AMP-activated protein kinase phosphorylation of endothelial NO synthase. *FEBS Lett*. 1999;443:285–289.
12. Khaliulin I, Clarke SJ, Lin H, Parker JE, Suleiman MS, Halestrap AP. Temperature preconditioning of isolated rat hearts - a potent cardioprotective mechanism involving a reduction in oxidative stress and inhibition of the mitochondrial permeability transition pore. *J Physiol*. 2007;581: H1147–H1161.
13. Dyck JR, Lopaschuk GD. AMPK alterations in cardiac physiology and pathology: Enemy or Ally? *J Physiol*. 2006;574:95–112.
14. Beauloye C, Marsin AS, Bertrand L, Krause U, Hardie DG, Vanoverschelde JL, Hue L. Insulin antagonizes AMP-activated protein kinase activation by ischemia or anoxia in rat hearts, without affecting total adenine nucleotides. *FEBS Lett*. 2001;505:348–352.
15. Oguchi T, Kashimoto S, Yamaguchi T, Nakamura T, Kumazawa T. Comparative effects of halothane, enflurane, isoflurane and sevoflurane on function and metabolism in the ischaemic rat heart. *Br J Anaesth*. 1995;74:569–575.
16. Bolli R, Jeroudi MO, Patel BS, Aruoma OI, Halliwell B, Lai EK, McCay PB. Marked reduction of free radical generation and contractile dysfunction by antioxidant therapy begun at the time of reperfusion. Evidence that myocardial "stunning" is a manifestation of reperfusion injury. *Circ Res*. 1989;65:607–622.
17. Nossuli TO, Hayward R, Scalia R, Lefer AM. Peroxynitrite reduces myocardial infarct size and preserves coronary endothelium after ischemia and reperfusion in cats. *Circulation*. 1997;96:2317–2324.
18. Maulik N, Yoshida T, Das DK. Oxidative stress developed during the reperfusion of ischemic myocardium induces apoptosis. *Free Radic Biol Med*. 1998;24:869–875.
19. Hawley SA, Pan DA, Mustard KJ, Ross L, Bain J, Edelman AM, Frenguelli BG, Hardie DG. Calmodulin-dependent protein kinase kinase-beta is an alternative upstream kinase for AMP-activated protein kinase. *Cell Metab*. 2005;2:9–19.
20. Depre C, Wang L, Sui X, Qiu H, Hong C, Hedhli N, Ginion A, Shah A, Pelat M, Bertrand L, Wagner T, Gaussin V, Vatner SF. H11 kinase prevents myocardial infarction by preemptive preconditioning of the heart. *Circ Res*. 2006;98:280–288.
21. Juhaszova M, Zorov DB, Kim SH, Pepe S, Fu Q, Fishbein KW, Ziman BD, Wang S, Ytrehus K, Antos CL, Olson EN, Sollott SJ. Glycogen synthase kinase-3beta mediates convergence of protection signaling to inhibit the mitochondrial permeability transition pore. *J Clin Invest*. 2004;113:1535–1549.
22. Toma O, Weber NC, Wolter JI, Obal D, Preckel B, Schlack W. Desflurane preconditioning induces time-dependent activation of protein kinase C epsilon and extracellular signal-regulated kinase 1 and 2 in the rat heart in vivo. *Anesthesiology*. 2004;101:1372–1380.
23. Novalija E, Kevin LG, Camara AK, Bosnjak ZJ, Kampine JP, Stowe DF. Reactive oxygen species precede the epsilon isoform of protein kinase C in the anesthetic preconditioning signaling cascade. *Anesthesiology*. 2003;99:421–428.
24. Raphael J, Rivo J, Gozal Y. Isoflurane-induced myocardial preconditioning is dependent on phosphatidylinositol-3-kinase/Akt signalling. *Br J Anaesth*. 2005;95:756–763.