Dark chocolate receptors: epicatechin-induced cardiac protection is dependent on δ-opioid receptor stimulation

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Dark chocolate receptors: epicatechin-induced cardiac protection is dependent on δ-opioid receptor stimulation. Am J Physiol Heart Circ Physiol 299: H1604–H1609, 2010. First published September 10, 2010; doi:10.1152/ajpheart.00073.2010.—Epicatechin, a flavonoid, is a well-known antioxidant linked to a variety of protective effects in both humans and animals. In particular, its role in protection against cardiovascular disease has been demonstrated by epidemiologic studies. Low-dose epicatechin, which does not have significant antioxidant activity, is also protective; however, the mechanism by which low-dose epicatechin induces this effect is unknown. Our laboratory tested the hypothesis that low-dose epicatechin mediates cardiac protection via opioid receptor activation. C57BL/6 mice were randomly assigned to 1 of 10 groups: control, epicatechin, naloxone (nonselective opioid receptor antagonist), epicatechin + naloxone, naltrindole (δ-specific opioid receptor antagonist), epicatechin + naltrindole, norbinaltorphimine (nor-BNI, κ-specific opioid receptor antagonist), epicatechin + nor-BNI, 5-hydroxydecanoic acid [5-HD, ATP-sensitive potassium channel antagonist], and epicatechin + 5-HD. Epicatechin (1 mg/kg) or other inhibitors (5 mg/kg) were administered by oral gavage or intraperitoneal injection, respectively, daily for 10 days. Mice were subjected to 30 min coronary artery occlusion followed by 2 h of reperfusion, and infarct size was determined via planimetry. Whole heart homogenates were assayed for downstream opioid receptor signaling targets. Infarct size was significantly reduced in epicatechin- and epicatechin + nor-BNI-treated mice compared with control mice. This protection was blocked by naloxone, naltrindole, and 5-HD. Epicatechin and epicatechin + nor-BNI increased the phosphorylation of Src, Akt, and IkBα, while simultaneously decreasing the expression of c-Jun N-terminal kinase and caspase-activated DNase. All signaling effects are consistent with opioid receptor stimulation and subsequent cardiac protection. Naloxone, naltrindole, and 5-HD attenuated these effects. In conclusion, epicatechin acts via opioid receptors and more specifically through the δ-opioid receptor to produce cardiac protection from ischemia-reperfusion injury.

ischemia-reperfusion

CARDIOVASCULAR DISEASE (CVD) is one of the leading causes of death in the United States. Many of the risk factors associated with CVD such as high blood cholesterol, obesity, and high blood pressure may take years to develop, which is why diet is vitally important to reduce the risks of developing CVD. Several epidemiologic studies show that these risk factors may be significantly reduced with a diet rich in flavonoids. Furthermore, these studies show an inverse correlation between the intake of flavonoid-rich cocoa products and mortality because of coronary heart disease (13a).

Epicatechin, a flavonoid that is a major component of cocoa and dark chocolate, is a well-known antioxidant associated with a lower risk of stroke and heart failure (8, 24, 30). Flavonoids, a major subclass of polyphenols that are well characterized for their antilipidemic and anti-inflammatory effects, are able to target a variety of cellular sites (28). Flavonoids also are associated with vasodilation, reduced platelet aggregation, and low-density lipoprotein oxidation (1). Studies show that 6.3 g (30 kCal) per day of dark chocolate containing 30 mg of polyphenols are sufficient to reduce blood pressure in hypertensive patients (31). Recent data provide evidence for the protective effect of epicatechin on vascular function and against myocardial ischemia-reperfusion injury (26, 36).

Several studies show a protective role of flavonoids at the membrane surface through interactions with both lipid and protein components of these membranes (13, 20). Previous studies showed the binding of flavonoids with various receptors: androgen and/or estrogen, insulin-like growth factor-1, epidermal growth factor, aryl hydrocarbon, Fas, vascular endothelial growth factor, and laminin (22). Flavonoids demonstrate both opioid receptor antagonist and agonist activity. Opioid receptors are a subset of key upstream receptors that induce cardiac protection from ischemia-reperfusion injury (12, 16, 23). Structure-activity relationships of flavonoids with opioid receptor ligands show binding activity in vitro (14), and flavonoids reduce morphine-induced opiate withdrawal (4–6, 32). Other groups have concluded that antinociception induced by flavonoids underlies the activation of the opioid system since this observed analgesic effect was reversed by naloxone (15). Collectively, such studies provide evidence for the interaction of flavonoids with opioid receptors. We propose that epicatechin has opioid receptor binding capacity and activates opioid receptor-mediated downstream signaling to modulate myocardial ischemia-reperfusion injury in vivo.

MATERIALS AND METHODS

Animals. All animals were treated in compliance with the Guide for the Care and Use of Laboratory Animals, and animal use protocols were approved by the Veterans Affairs San Diego Healthcare System Institutional Animal Care and Use Committee (San Diego, CA). C57BL/6 male mice (aged 8–10 wk, and 24–26 g body wt) were purchased from Jackson Laboratories (Bar Harbor, ME). The animals were kept on a 12-h:12-h light-dark cycle in a temperature- and humidity-controlled room.

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Experimental design. Mice were randomly assigned to the following 10 groups: control, (–)-epicatechin, naloxone, epicatechin + naloxone, naltrindole, epicatechin + naltrindole, norbinaltorphimine (nor-BNI), epicatechin + nor-BNI, 5-hydroxydecanoic acid (5-HD), or epicatechin + 5-HD. Control animals were given 0.3 ml of saline (vehicle) by oral gavage for 10 days. Epicatechin was administered by oral gavage daily (1 mg/kg body wt, dissolved in 0.3 ml of saline) for 10 days. Naloxone, naltrindole, nor-BNI, and 5-HD were administered by intraperitoneal injection daily (5 mg/kg body wt, dissolved in 0.3 ml of saline) for 10 days and 2 h before ischemia-reperfusion.

In vivo ischemia-reperfusion experimental protocol. Surgery was performed as previously described (34). Briefly, mice were anesthetized with pentobarbital sodium (80 mg/kg) and mechanically ventilated. Ischemia was produced by occluding the left coronary artery with a 7-0 silk suture on a tapered BV-1 needle (Ethicon) for 30 min. A small piece of polyethylene tubing was used to secure the silk ligature without damaging the artery. After 30 min of occlusion, the ligature was released, and the heart was reperfused for 2 h. Reperfusion was confirmed by observing return of blood flow in the epicardial coronary arteries.

Infarct size. The area at risk was determined by staining with 1% Evans blue (1.0 ml, Sigma). The heart was immediately excised and cut into 1-mm slices (McIlwain tissue chopper; Brinkmann Instruments). The left ventricle was counterstained with 1% 2,3,5-triphenyltetrazolium chloride (Sigma). The images were analyzed by ImagePro-Plus (Media Cybernetics), and infarct size was determined by planimetry as described previously (34).

Immunoblot. In a separate group of mice, left ventricular whole tissue lysates were prepared for analysis of downstream signaling molecules. Proteins were separated by SDS-polyacrylamide gel electrophoresis using 10% polyacrylamide precast gels (Invitrogen) and transferred to polyvinylidene difluoride membranes by electroelution. Membranes were blocked in 20 mM TBS-Tween (1%) containing 3% bovine serum albumin and incubated overnight at 4°C with primary antibody containing phospho-(p)-Src, Src, p-Akt, Akt, p-JNK, JNK, pIkBo, IkB, caspase-activated DNase (CAD), and GAPDH, which served as the loading control for JNK and CAD. The phosphorylated proteins were normalized to respective total protein blots. The blots were visualized using corresponding secondary antibodies conjugated with horseradish peroxidase (Santa Cruz Biotechnology) and enhanced chemiluminescence reagent (GE Healthcare).

Echocardiography. Echocardiography was performed in mice (which were subsequently used for immunoblot analysis) anesthetized with isoflurane (1% in oxygen) using a Philips, Sonos 5500. The following parameters were measured or estimated from a M-mode short-axis view of the left ventricle during systole and diastole: left ventricular internal diameter, interventricular septal dimension, posterior wall dimension, percent fractional shortening, and left ventricular mass.

Statistical analysis. Data analysis was performed by observers blinded to the experimental groups. Statistical analyses were performed by one-way ANOVA followed by post hoc unpaired Student’s t-test with Bonferroni correction for multiple comparisons. Values are expressed as means ± SD. Statistical significance was defined as \( P < 0.05 \).

RESULTS

Mice were administered vehicle, epicatechin, naloxone, naltrindole, nor-BNI, 5-HD, or a combination of epicatechin with inhibitors for 10 days and then exposed to 30 min of coronary artery occlusion, followed by 2 h of reperfusion. Infarct size was then evaluated. The area at risk as a percentage of the left ventricle was similar among groups (Fig. 1A). Infarct size was significantly reduced in epicatechin- and epicatechin + nor-BNI-treated mice compared with the control mice (Fig. 1B).

Epicatechin-induced protection was attenuated by naloxone, naltrindole, and 5-HD; however, these pharmacological antagonists alone did not have an effect on infarct size. Nor-BNI did not block the protective effect of epicatechin. These data suggest that the protective effect of epicatechin is mediated by the \( \delta \)-opioid receptor.

In another group of mice, hearts were removed after 10 days of vehicle, epicatechin, inhibitors, or combination of epicatechin + inhibitors administration and underwent biochemical analysis. Epicatechin- and epicatechin + nor-BNI-treated animals showed a significant increase in p-Src, p-Akt, and p-IkBo protein compared with controls (Fig. 2). JNK and CAD...
expression was significantly reduced in epicatechin and epicatechin + nor-BNI (Fig. 3). The change in signaling protein expression and the phosphorylation induced by epicatechin was attenuated by treatment with naloxone, naltrindole, and 5-HD (Figs. 2 and 3). To determine whether the epicatechin, opioid receptor antagonists, and/or ATP-sensitive potassium (K\textsubscript{ATP}) channel blocker would affect the physiological phenotype, transthoracic echocardiography was performed. All the observed parameters were unchanged (Table 1).

**DISCUSSION**

The results of the present study indicate that the ability of epicatechin to elicit survival signaling in the mouse heart can be modulated by inhibiting δ-opioid receptors. This is the first demonstration of a receptor-mediated mechanism for epicatechin-induced cardiac protection. Human dietary intervention trials with flavonoid-containing cocoa products have demonstrated that a daily consumption of flavonoid-containing dark chocolate is associated with a significant reduction in systolic blood pressure and improvements in endothelial and platelet function (9). In a double-blind, randomized study, the effect of flavonoid-rich dark chocolate compared with cocoa-free control chocolate on coronary vasomotion in cardiac transplant recipients showed that consuming dark chocolate induced coronary vasodilation, improved coronary vascular function, and decreased platelet adhesion (10). These beneficial effects were paralleled with serum epicatechin concentrations (26). Such clinical data suggest that cacao ingestion has significant potential for lowering cardiovascular risk; however, the specific mechanism to produce these effects is not yet clearly understood.

Previous studies show that opioid agonists and volatile anesthetics mediate cardioprotection by the activation of opioid receptors (21, 27). Opioid receptors are involved in cardiac protection via the activation of Akt and Src pathways (7, 11), and epicatechin activates similar protein kinase signaling pathways as opioids (25). Our study validates other findings that epicatechin induces Akt, Src, and I\textsubscript{B} phosphorylation in the heart. A number of recent studies have determined an essential role for Src/phosphatidylinositol 3-kinase/Akt pathway in myocardial survival following ischemia-reperfusion injury, and Akt also functions as a nodal point to coordinate growth factor signaling with myocyte survival (18). The phosphorylation of I\textsubscript{B} is a prerequisite for NF-κB activation and is a surrogate marker of the induction of the survival NF-κB pathway. Increased binding of NF-κB to DNA protects myocytes from ischemic stress (17). NF-κB increases and JNK decreases cell survival in animal models (2); we show that epicatechin reduces apoptosis via decreased JNK. 3'–O-methyl epicatechin, which cannot serve as an antioxidant, produces a similar antiapoptotic effect as epicatechin, suggesting that antioxidant activity is not the primary mechanism of protection (29).

Naloxone is a competitive, nonselective antagonist of opioid receptors, whereas naltrindole and nor-BNI are selective δ- and γ-opioid receptor antagonists. Immunoblot of phosphorylated survival kinase in whole heart homogenates from control and Epi-, Nal-, Epi + naloxone-, naltrindole-, Epi + naltrindole-, nor-BNI-, Epi + nor-BNI-, 5-HD-, and Epi + 5-HD-administered animals. Epi and Epi + nor-BNI showed a significant increase in phospho (p)-Src (A), p-Akt (B), and p-I\textsubscript{B}α (C), and this increased phosphorylation was attenuated by Nal, naltrindole, and 5-HD. Densitometry was normalized to expression of respective nonphosphorylated proteins. *P < 0.05 vs. control; n = 5/group.

![Fig. 2. Increased survival kinase expression by Epi was attenuated by opioid receptor antagonism. Immunoblot of phosphorylated survival kinase in whole heart homogenates from control and Epi-, Nal-, Epi + naloxone-, naltrindole-, Epi + naltrindole-, nor-BNI-, Epi + nor-BNI-, 5-HD-, and Epi + 5-HD-administered animals. Epi and Epi + nor-BNI showed a significant increase in phospho (p)-Src (A), p-Akt (B), and p-I\textsubscript{B}α (C), and this increased phosphorylation was attenuated by Nal, naltrindole, and 5-HD. Densitometry was normalized to expression of respective nonphosphorylated proteins. *P < 0.05 vs. control; n = 5/group.](image-url)
κ-opioid receptor antagonists, respectively. Naloxone and naltrindole attenuated the cardiac protective effects of epicatechin, blocked the induction of phosphoproteins involved in cardiac protection, and reversed the effect of epicatechin on apoptotic proteins. The specific κ-opioid receptor antagonist, nor-BNI, did not block epicatechin-induced cardiac protection. Opioid peptide-mediated cardiac protection also involves mitochondrial K_ATP channels in cardiac myocytes (3); we show that epicatechin-induced protection is attenuated by 5-HD. This may suggest why flavonoids exert cardiac protective effects by partial mitochondrial uncoupling in isolated mitochondria (33).

Fig. 3. Decreased apoptotic protein expression by Epi was attenuated by opioid receptor antagonism. Immunoblot analysis of expression of JNK and caspase-activated DNase (CAD) in control and Epi-, Nal-, Epi + Nal-, naltrindole-, Epi + naltrindole-, nor-BNI-, Epi + nor-BNI-, 5-HD-, and Epi + 5-HD-administered mice. Densitometry was normalized to GAPDH. Epi and Epi + nor-BNI significantly decreased the expression of JNK and CAD; no differences in JNK and CAD expression were observed between control and other groups. *P < 0.05 vs. control; n = 5/group.

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Values are means ± SD; n = 5/group. Epi, epicatechin; Nal, naloxone; 5-HD, 5-hydroxydecanoic acid; nor-BNI, norbinaltorphimine; LVIDd, left ventricular internal cavity diameter in diastole; LVIDs, left ventricular internal cavity diameter in systole; ISDd, interventricular septal dimension in diastole; ISDs, interventricular septal dimension in systole; LVPWDd, left ventricular posterior wall dimension in diastole; LVPWDs, left ventricular posterior wall dimension in systole; LVM, left ventricular mass.
Epicatechin in the present study was given at a low dose (1 mg/kg) with long-term administration (10 days). At this dose, an antioxidant effect that occurs with high-dose flavonoid administration likely does not occur (35). With our treatment regimen, we observed a potent protective effect, indicating that there are likely two mechanisms for flavonoids to promote cell survival: 1) antioxidant activity and/or 2) signaling via receptor-mediated pathways to activate survival signaling.

Our findings should be interpreted within the constraints of potential limitations. It is possible that the protective effects of epicatechin involved changes in blood pressure, but we did not specifically measure hemodynamic parameters in our treatment groups. However, a recent study using the exact parameters of dose, duration, and route of administration of epicatechin in rats showed no effect on blood pressure (36). Additionally, confirmatory studies could be performed to directly administer dark chocolate to mice instead of purified epicatechin and determine the dependence of this protection on opioid receptor stimulation, though it may be difficult in such a setting to guarantee equal dosage among animals of the active flavonoids.

In conclusion, we show that subantioxidant doses of epicatechin produce cardiac protection via σ-opioid receptor stimulation and activation of downstream survival and antiapoptotic pathways, thus defining a receptor-mediated mechanism for epicatechin action. Epicatechin-induced cardiac protection also appears to involve the activation of the mitochondrial K$_{\text{ATP}}$ channel. These data suggest the intriguing possibility that the opioid modulatory and mitochondrial effects of flavonoids may be linked and suggest avenues for future investigations.

DISCLOSES

No conflicts of interest (financial or otherwise) are declared by the author(s).

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