

Involvement of Protein Kinase A in Cannabinoid Receptor-Mediated Protection from Oxidative Neuronal Injury

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ABSTRACT

CB1 cannabinoid receptors (CB1Rs) are involved in protecting the brain from ischemia and related disorders. However, the underlying protective mechanisms are incompletely understood. We investigated the effect of CB1R activation on oxidative injury, which has been implicated in neuronal death after cerebral ischemia and neurodegenerative disorders, in mouse cortical neuron cultures. The CB1R agonist Win 55212-2 [*R*-(+)-[2,3-dihydro-5-methyl-3-[(morpholinyl)methyl]pyrrolo[1,2,3-*de*]-1,4-benzoxazin-yl]-(1-naphthalenyl)methanone mesylate] reduced neuronal death, measured by lactate dehydrogenase release, in cultures treated with 50 μ M FeCl₂, and its protective effect was attenuated by the CB1R antagonist SR141716A [*N*-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide hydrochloride]. The endocannabinoid anandamide reproduced the effect of Win 55212-2, as did the antioxidant 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox). Neuronal injury was more severe after *in vitro* or *in vivo* administration of FeCl₂ to CB1R-knockout compared with wild-type mice. Win 55212-2 reduced the formation of reactive oxidative species in cortical

neuron cultures treated with FeCl₂, consistent with an antioxidant action. Pertussis toxin reduced CB1R-mediated protection, which points to a protective mechanism that involves signaling through G_{i/o} proteins. Since CB1R-activated G protein signaling inhibits protein kinase A but activates phosphatidylinositol 3-kinase (PI3K), we tested the involvement of these pathways in CB1R-mediated neuroprotection. Dibutyl-cyclic adenosine monophosphate (dbcAMP) blocked protection by Win 55212-2, whereas the PI3K inhibitor wortmannin did not, and the effect of dbcAMP was inhibited by the protein kinase A inhibitor H89 [*N*-[2-((*p*-bromocinnamyl)amino)ethyl]-5-isoquinolinesulfonamide] (≥ 10 nM). CB1R-induced, SR141716A-, pertussis toxin-, and dbcAMP-sensitive protection was also observed for two other oxidative insults, exposure to H₂O₂ or buthionine sulfoximine. Therefore, receptor-stimulated inhibition of protein kinase A seems to be required for the neuroprotective effect of CB1R activation against oxidative neuronal injury.

Endogenous cannabinoid (endocannabinoid) signaling pathways, consisting of endocannabinoids such as anandamide and 2-arachidonylglycerol and the G protein-coupled CB1R and CB2R receptors, have been implicated in a range of physiological brain functions. In addition, endocannabinoid signaling provides neuroprotection after ischemia and other cerebral insults. Cannabinoid receptor agonists reduce neuronal loss from global and focal cerebral ischemia (Nagayama et al., 1999) and brain trauma (Panikashvili et al., 2001), and the size of cerebral infarcts after middle cerebral artery occlusion is increased in CB1R-knockout mice (Parmentier-

Batteur et al., 2002). The brain's response to ischemia involves up-regulation of neuronal CB1 receptors (Jin et al., 2000) and increased production of endocannabinoid-related compounds that modulate the inflammatory response to ischemia (Franklin et al., 2003). Therefore, endogenous cannabinoid signaling mechanisms may represent a key component of protection and repair programs mobilized in the injured brain.

Cannabinoid receptors are coupled to a variety of downstream signal transduction pathways. CB1Rs are located primarily on presynaptic nerve terminals, where they interact with heterotrimeric G proteins (especially G_{i/o}), releasing G α subunits and G $\beta\gamma$ dimers (Herlitz et al., 1996; Ikeda, 1996). This results in inhibition of adenylyl cyclase, reduced levels of cyclic AMP, and decreased activation of protein kinase A (Childers and Deadwyler, 1996), as well as dimin-

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ABBREVIATIONS: CB1R, CB1 cannabinoid receptor; PI3K, phosphatidylinositol 3-kinase; ROS, reactive oxidative species; *R*-(+)-Win 55212-2 mesylate, (+)-[2,3-dihydro-5-methyl-3-[(morpholinyl)methyl]pyrrolo[1,2,3-*de*]-1,4-benzoxazin-yl]-(1-naphthalenyl)methanone mesylate; SR141716A, *N*-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide hydrochloride; Trolox, (\pm)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid; BSO, DL-buthionine-[S,R]-sulfoximine; H89, *N*-[2-((*p*-bromocinnamyl)amino)ethyl]-5-isoquinolinesulfonamide; dbcAMP, dibutyl-cyclic adenosine monophosphate; LDH, lactate dehydrogenase; NMDA, *N*-methyl-D-aspartate; ANOVA, analysis of variance.

ished Ca^{2+} influx through voltage-gated Ca^{2+} channels (Mackie and Hille, 1992). Ca^{2+} -dependent vesicular release of neurotransmitters, including GABA and glutamate, is thereby uncoupled from nerve terminal depolarization and decreased. Cannabinoids also activate protein kinase signaling pathways involving mitogen-activated protein kinase kinase/extracellular signal-regulated kinase (Bouaboula et al., 1995; Galve-Roperh et al., 2002; Derkinderen et al., 2003), p38 (Derkinderen et al., 2001), and c-Jun N-terminal kinase (Rueda et al., 2000), as well as phosphatidylinositol 3-kinase (PI3K)/Akt (Gomez del Pulgar et al., 2000) and focal adhesion kinase (Derkinderen et al., 1996). However, which of these pathways are important for cannabinoid-induced neuroprotection in stroke and other settings is unclear.

To begin to address this issue, we investigated the possible involvement of protein kinase A inhibition as a mechanism for CB1R-mediated neuroprotection in neuronal cultures. The neurotoxic insult we used was FeCl_2 -induced oxidative injury, because this form of injury has been implicated in neuronal death from focal ischemia with reperfusion (White et al., 2000; Schaller and Graf, 2004), as occurs in patients with stroke. Fe^{2+} accumulation, leading to increased generation of reactive oxidative species (ROS) and oxidative cell damage, has also been implicated in neurodegenerative disorders, including Alzheimer's disease and Parkinson's disease (Zecca et al., 2004), suggesting that it may represent a more widespread neurotoxic process. Our results indicate that the neuroprotective antioxidant effect of cannabinoids, acting through CB1R and $\text{G}_{i/o}$ proteins, depends on suppression of cyclic AMP signaling through protein kinase A.

Materials and Methods

Drugs. *R*-(+)-[2,3-Dihydro-5-methyl-3-[(morpholinyl)methyl]-pyrrolo[1,2,3-*de*]-1,4-benzoxazin-yl)-(1-naphthalenyl)methanone mesylate [*R*-(+)-Win 55212-2 mesylate] was purchased from Sigma/RBI (Natick, MA) and *N*-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide hydrochloride (SR141716A) was obtained from the National Institute on Drug Abuse (Bethesda, MD). Anandamide was purchased from Tocris Cookson Inc. (Ellisville, MO), and ferrous chloride (FeCl_2), (\pm)-6-hydroxy-2,5,7,8-tetramethyl chromane-2-carboxylic acid (Trolox), DL-buthionine-[S,*R*]-sulfoximine (BSO), and *N*-[2-(*p*-bromocinnamyl)amino]ethyl-5-isoquinolinesulfonamide (H89) were purchased from Sigma-Aldrich (St. Louis, MO). Pertussis toxin, adenosine 3',5'-cyclic monophosphate dibutyryl sodium salt (dbcAMP) were obtained from Calbiochem (San Diego, CA), and wortmannin was from Roche Diagnostics (Indianapolis, IN).

Primary Cortical Cell Culture. Neuron-enriched mouse cerebral cortical cultures were prepared from the brains of day E16 wild-type CD1 and CB1 knockout mice. Neocortex was triturated, and dissociated cells were plated at five hemicortices per 24-well plastic culture plate in Eagle's minimal essential medium (Earle's salts, supplied glutamine-free) supplemented with 5% horse serum, 5% fetal bovine serum, 21 mM glucose, 26.5 mM bicarbonate, and 2 mM L-glutamine. Cultures were maintained at 37°C in a humidified 5% CO_2 incubator, and beginning 2 days after plating, cultures were given fresh medium lacking fetal serum twice weekly. Cytosine arabinoside (10 μM) was added for days 5 to 7 in vitro.

Measurement of Cell Death. Between days 12 and 14 in vitro, cultures were rinsed with serum-free minimal essential medium and treated for 24 h with FeCl_2 , BSO, or H_2O_2 , with or without other drugs. Cell death was quantified by measuring lactate dehydrogenase (LDH) release into the bathing medium over 24 h and expressed as a percentage of cell death induced by 500 μM *N*-methyl-D-aspar-

tate (NMDA): $(\text{LDH} - \text{LDH}_{\text{control}})/(\text{LDH}_{\text{NMDA}} - \text{LDH}_{\text{control}}) \times 100\%$. In some experiments, cell death measurements were confirmed by trypan blue exclusion. Trypan blue dye (0.08%) was added to cultures for 5 min at 25°C, buffer was replaced with dye-free buffer, and dye-containing (injured) and dye-excluding (viable) cells were counted in an average of five 40 \times microscope fields per well.

Intracerebral Injection of FeCl_2 . Oxidative injury was induced in vivo as described previously (Won et al., 2000) by injection of 20 nmol of FeCl_2 in 1 μl of sterile phosphate-buffered saline into the parietal cortex at a site 1.5 mm caudal to bregma, 3.0 mm from the midline, and 0.8 mm below the dural surface. After 24 h, 30- μm coronal brain sections were stained with hematoxylin to delineate the resulting lesion.

Detection of Oxidative Activity. Cultures were loaded with 5 μM hydroethidine (Molecular Probes, Eugene, OR) in HEPES-buffered control salt solution (HCSS) containing 120 mM NaCl, 5 mM KCl, 1.6 mM MgCl_2 , 2.3 mM CaCl_2 , 15 mM glucose, 20 mM HEPES, and 10 mM NaOH. Cultures were incubated for 20 min at 37°C and washed three times with HEPES-buffered control salt solution. The fluorescence signal of oxidized hydroethidine was observed with a Nikon E800 fluorescence microscope at excitation 510 to 550 nm and emission >580 nm.

Immunocytochemistry. Cultures were fixed in 4% paraformaldehyde for 30 min, incubated in 10% goat serum for 1 h, and immunolabeled with a mouse monoclonal antibody against NeuN (1:200; Chemicon International, Temecula, CA) at 4°C overnight. Cultures were washed with phosphate-buffered saline and reacted with fluorescein isothiocyanate-conjugated anti-mouse IgG (1:200; Vector Laboratories, Burlingame, CA) for 1 h. The fluorescence signals were detected at excitation 470 nm and emission 505 nm.

Data Analysis. Data were expressed as mean \pm S.E.M. ANOVA and Student-Newman-Keuls test (multiple comparisons) or Student's *t* test (single comparisons) was used for statistical analysis, with *P* < 0.05 considered significant.

Results

To quantify LDH release, which was used as an index of cell death in our cortical neuron cultures, we defined release over 24 h in the absence of drugs as 0% and release over the same period in the presence of 500 μM NMDA as 100%. In our NMDA-sensitive neuronal cultures, LDH release, expressed as a percentage of NMDA-induced LDH release, is approximately equal to percentage of cell death. FeCl_2 (50 μM) caused the release of ~70% of LDH (Fig. 1A). This was reduced in the presence of increasing concentrations of the synthetic cannabinoid receptor agonist Win 55212-2, with 50% inhibition of the effect of FeCl_2 at 100 nM Win 55212-2. The endogenous (endo)cannabinoid agonist anandamide also decreased FeCl_2 toxicity, producing half-maximal inhibition at 300 nM anandamide (Fig. 1B). To determine which cannabinoid receptor mediated the protective effects of Win 55212-2 and anandamide, we treated some cultures with the CB1R antagonist SR141716A (1 μM), which by itself had no effect on FeCl_2 toxicity. SR141716A prevented the effects of both cannabinoid receptor agonists, implicating CB1R. Trolox, a water-soluble vitamin E analog and antioxidant, abolished FeCl_2 toxicity, consistent with involvement of oxidative injury in the observed cell death. The protective effect of Win 55212-2 against FeCl_2 toxicity was confirmed by trypan blue exclusion (Fig. 1C).

To confirm the involvement of CB1Rs in the regulation of FeCl_2 toxicity, we compared the effects of increasing concentrations of FeCl_2 on LDH release in cultures prepared from wild-type and CB1R-knockout mice. The toxic effect of FeCl_2

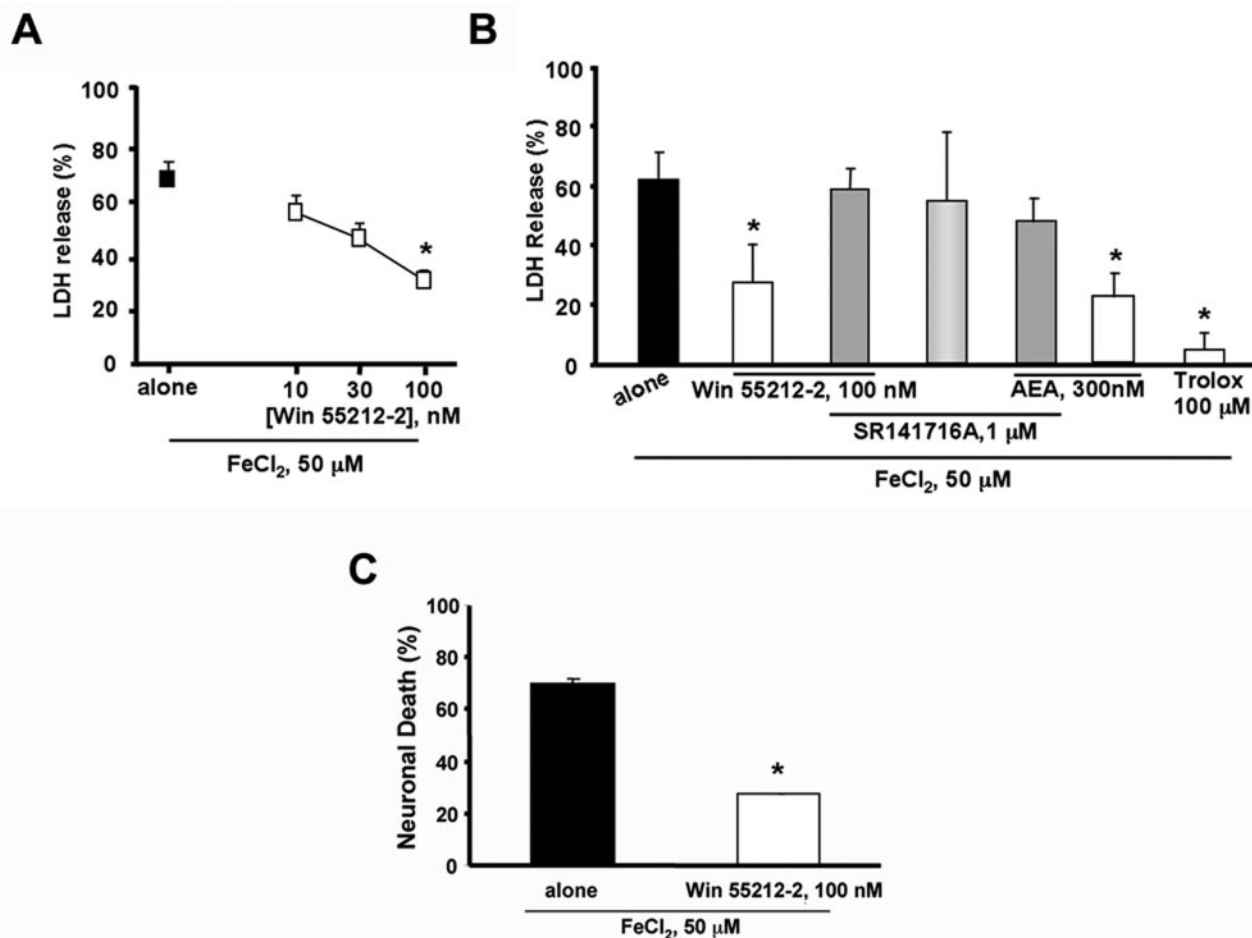


Fig. 1. CB1 receptor-mediated protection of cultured neurons from oxidative neuronal injury. A and C, cortical neuron cultures (12–15 days in vitro) were exposed to FeCl_2 for 24 h, in the absence and presence of Win 55212-2. B, cultures were exposed to FeCl_2 for 24 h, without drugs or with Win55212-2, SR141716A, anandamide, or Trolox. Cell death was measured by LDH efflux into the medium (A and B) or trypan blue exclusion (C). Data shown are means \pm S.E.M. from 16 to 20 wells per condition. *, $P < 0.05$ compared with FeCl_2 alone (ANOVA and Student-Newman-Keuls tests).

was potentiated by CB1R deletion (Fig. 2A), implying that endogenous cannabinoid signaling through this receptor serves to mitigate FeCl_2 -induced injury. CB1R-knockout mice also showed an increase in lesion size compared with wild-type mice after intracerebral injection of FeCl_2 in vivo (Fig. 2, B and C).

Because some downstream effects of CB1R activation result from coupling to inhibitory G proteins (G_i), we next examined the effect of inhibiting G_i . Pertussis toxin, which catalyzes the ADP ribosylation of α_i and uncouples G_i from interacting receptors, abolished Win 55212-2-mediated protection from FeCl_2 toxicity (Fig. 3). This was not a result of pertussis toxin toxicity because pertussis toxin alone had little or no effect on LDH release. Therefore, the protective effect of Win 55212-2 seems to require G_i .

CB1R-stimulated, G_i -dependent effects are associated with several signal transduction pathways. Among these, one of the best characterized involves the inhibition of adenylyl cyclase, resulting in decreased production of cyclic AMP and reduced activation of cyclic AMP-dependent protein kinase, or protein kinase A. However, other protein kinases, including PI3K, also mediate some G_i -dependent effects of CB1R stimulation. Whereas protein kinase A activity is diminished by CB1R acting via G_i , PI3K activity is enhanced. To test whether either of these protein kinases might be involved in

neuroprotection by cannabinoids, we measured FeCl_2 -induced LDH release in the presence of Win 55212-2, together with either dbcAMP, which activates protein kinase A directly, or wortmannin, an inhibitor of PI3K. dbcAMP reversed the protective effect of Win 55212-2, consistent with involvement of protein kinase A, whereas wortmannin was ineffective (Fig. 4), implying that activation of PI3K did not contribute to neuroprotection. Because cAMP can interact with targets other than protein kinase A, we also examined the effect of the membrane-permeable protein kinase A inhibitor H89 on the reversal of cannabinoid protection by dbcAMP. In the presence of dbcAMP, H89 restored the protective effect of Win 55212-2. This occurred at concentrations consistent with selective inhibition of protein kinase A (half-maximal effect at ~ 10 nM) and much lower than are associated with effects on other substrates (Penn et al., 1999; Davies et al., 2000).

To ascertain whether Win 55212-2-induced, SR141716A-, pertussis toxin-, and dbcAMP-sensitive protection occurs for other types of oxidative injuries, we examined the effects of these agents in cultures treated with H_2O_2 or BSO. As shown in Fig. 5, the same CB1R, G_i , and protein kinase A effects observed for FeCl_2 toxicity seem to apply for H_2O_2 and BSO as well.

Fe^{2+} released from FeCl_2 causes free radical-induced cell

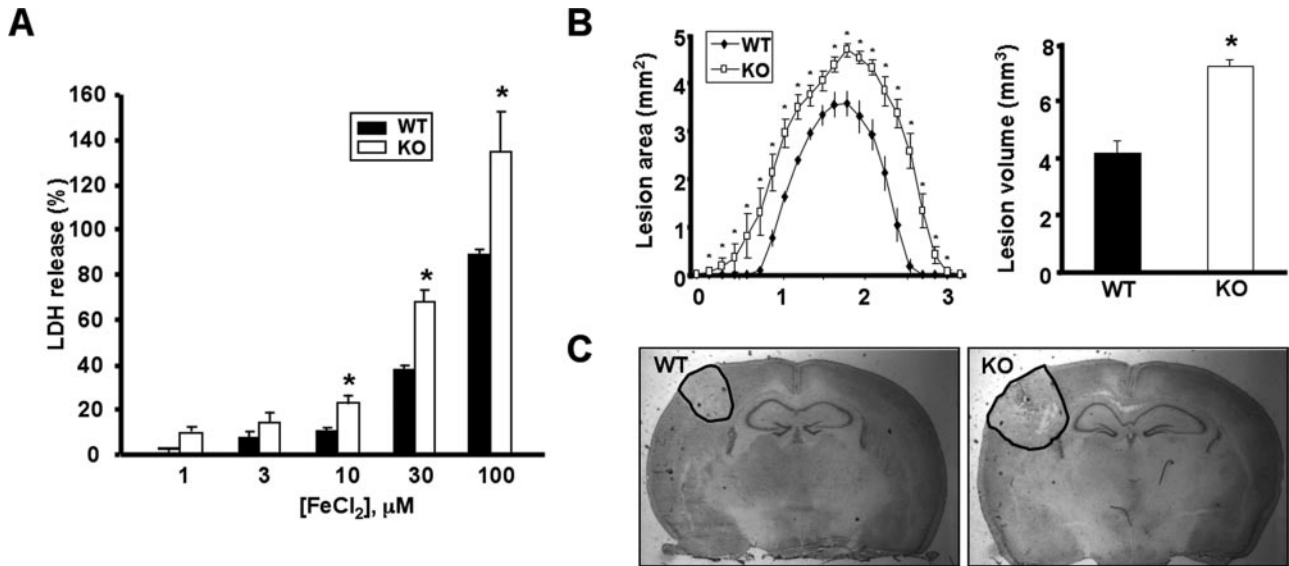


Fig. 2. Increased FeCl₂-induced injury in CB1R-knockout mice. A, cortical neuron cultures from wild-type (WT) and CB1R-knockout (KO) mice were exposed to FeCl₂ for 24 h, and cell death was measured by LDH efflux into the medium. Data shown are means ± S.E.M. from 16 wells per condition. *, *P* < 0.05 compared with WT (ANOVA and Student-Newman-Keuls tests). B, FeCl₂ (20 nmol) was injected into the parietal cortex of WT and KO mice; the area of the resulting hematoxylin-unstained lesion was measured at multiple coronal levels (left), and the volume (right) was calculated. Data shown are means ± S.E.M. from three (WT) or four (KO) mice per condition. *, *P* < 0.05 compared with WT (left, two-tailed Student's *t* test; right, Student's *t* test). C, representative WT and KO brains show FeCl₂-induced lesions of different size (outlined areas).

injury when it reacts with H₂O₂ via the Fenton reaction to generate OH[•]. To determine whether the protein kinase A-dependent protective effect of cannabinoids against FeCl₂ was accompanied by suppression of the production of ROS, we measured oxidative activity in our cultures with hydroethidine. Fluorescence photomicrographs showed increased fluorescence of ethidium, the oxidative product of hydroethidine, in cultures exposed to FeCl₂ (Fig. 6). Fluorescence was associated with neuronal nuclei, as shown by its colocalization with the neuronal nuclear marker NeuN. Win 55212-2 decreased fluorescence and its effect was counteracted by dbcAMP. Thus, Win 55212-2 produced parallel, protein ki-

nase A-dependent inhibition of FeCl₂ toxicity and FeCl₂-induced oxidative activity in our neuronal cultures.

Discussion

The major finding of this study is that cannabinoids protect cultured cortical neurons from FeCl₂-induced oxidative cell death, as well as from BSO and H₂O₂ toxicity, by a CB1R- and protein kinase A-dependent mechanism. Iron-mediated toxicity has been implicated in a spectrum of neurodegenerative disorders, based partly on the finding that brain iron

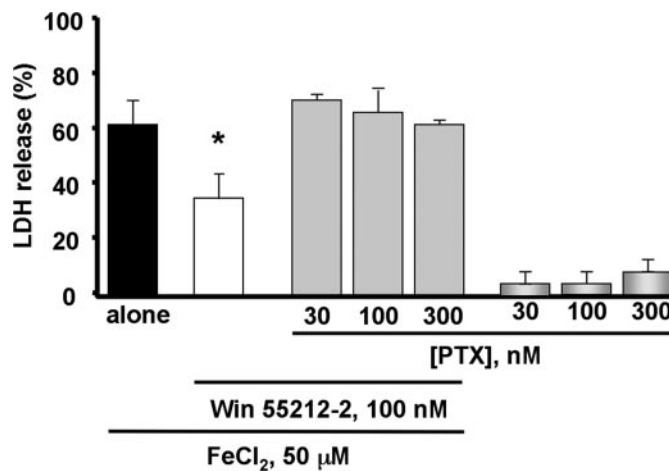


Fig. 3. Involvement of G_{i/o} proteins in the neuroprotective effect of Win 55212-2. Cortical neuron cultures were exposed to FeCl₂ for 24 h, in the absence and presence of Win 55212-2 or Win 55212-2 plus pertussis toxin (PTX). To control for possible direct toxic effects of PTX, some cultures were exposed to PTX alone. Cell death was measured by LDH efflux into the medium. Data shown are means ± S.E.M. from eight wells per condition. *, *P* < 0.05 compared with FeCl₂ alone (ANOVA and Student-Newman-Keuls tests).

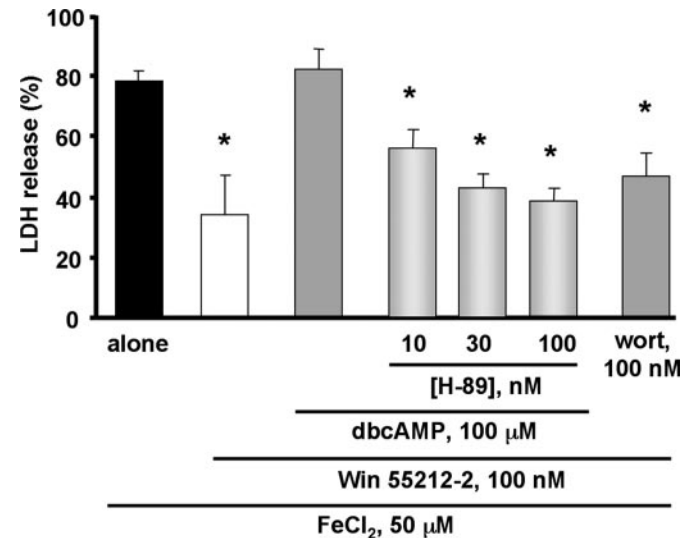


Fig. 4. Dependence of Win 55212-2-induced neuroprotection on inhibition of PKA. Cortical neuron cultures were exposed to FeCl₂ for 24 h, in the absence and presence of Win 55212-2, Win 55212-2 plus dbcAMP or wortmannin, or Win 55212-2 plus dbcAMP plus H89. Cell death was measured by LDH efflux into the medium. Data shown are means ± S.E.M. from 16 wells per condition. *, *P* < 0.05 compared with FeCl₂ alone (ANOVA and Student-Newman-Keuls tests).

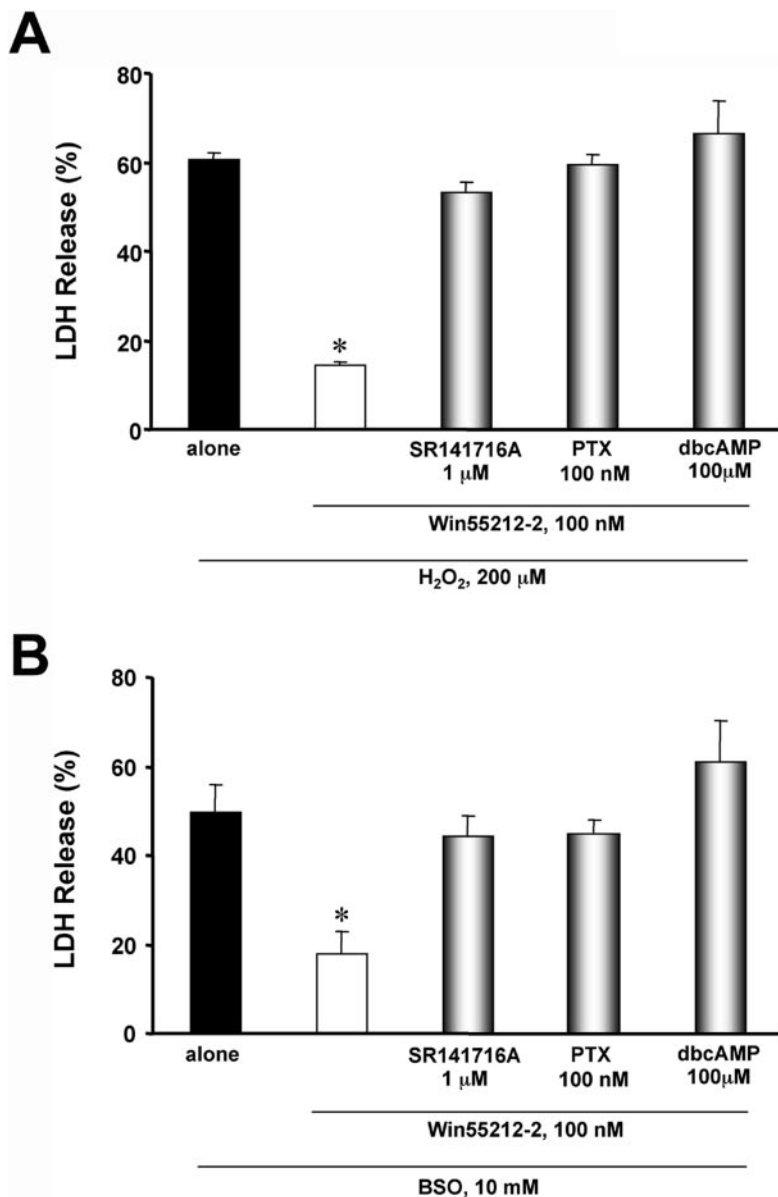


Fig. 5. Effects of Win55212-2, SR141716A, pertussis toxin (PTX), and dbcAMP on H₂O₂ and BSO toxicity. H₂O₂ or BSO was added to cortical neuron cultures, and 24 h later, cell death was measured by LDH efflux into the medium. Data shown are means \pm S.E.M. from 16 wells per condition. *, $P < 0.05$ compared with H₂O₂ or BSO alone (ANOVA and Student-Newman-Keuls tests).

levels are high in vulnerable brain regions and are increased further in these diseases (Zecca et al., 2004). The iron concentration that we used is similar to that used in other in vitro studies of iron neurotoxicity (Liu et al., 2003) and within the range of concentrations found in mouse and human brain (Griffiths et al., 1999; Moos et al., 2000; Zecca et al., 2001; Hajek et al., 2004).

Oxidative injury has been implicated previously in ischemic neuronal death that occurs during reperfusion, when ROS are generated (Schaller and Graf, 2004). The participation of oxidative mechanisms in FeCl₂ toxicity in our cultures was demonstrated by hydroethidine fluorescence and by the prevention of toxicity by the antioxidant Trolox. Evidence for the involvement of CB1R in cannabinoid neuroprotection in our cultures included the protective effects of both Win 55212-2 and anandamide, the ability of SR141716A to inhibit protection, and the more pronounced cell death observed in CB1R-knockout mice. The downstream signaling pathway through which CB1R-mediated protection is transduced was identified based on pertussis toxin sensitivity, pointing to a

G_{i/o}-based mechanism, and H89-sensitive inhibition by dbcAMP, indicating that a reduction in cAMP, leading to reduced activation of protein kinase A, was required. Neuroprotective effects of G_i-coupled receptor agonists have been reported previously for dopamine D1 (Noh and Gwag, 1997), δ -opioid (Tsao et al., 1998), and EP3 prostanoid (Bilak et al., 2004) receptors.

Prior studies on cannabinoids and oxidative neuronal injury have produced conflicting results. Hampson et al. (1998) reported that in cortical neurons cultures, antioxidant effects were responsible for nonreceptor-mediated neuroprotection from glutamate toxicity by cannabinoids. Marsicano et al. (2002) compared the ability of cannabinoids to protect against H₂O₂ in a stably transfected hippocampal cell line with and without CB1R, and in cerebellar granule cells from wild-type and CB1R-knockout mice, and also found that the antioxidant effects of cannabinoids were not CB1R-dependent. Antioxidative cytoprotection by cannabinoids was also observed in non-neuronal cells lacking cannabinoid receptors (Chen and Buck, 2000). However, in the retina, where

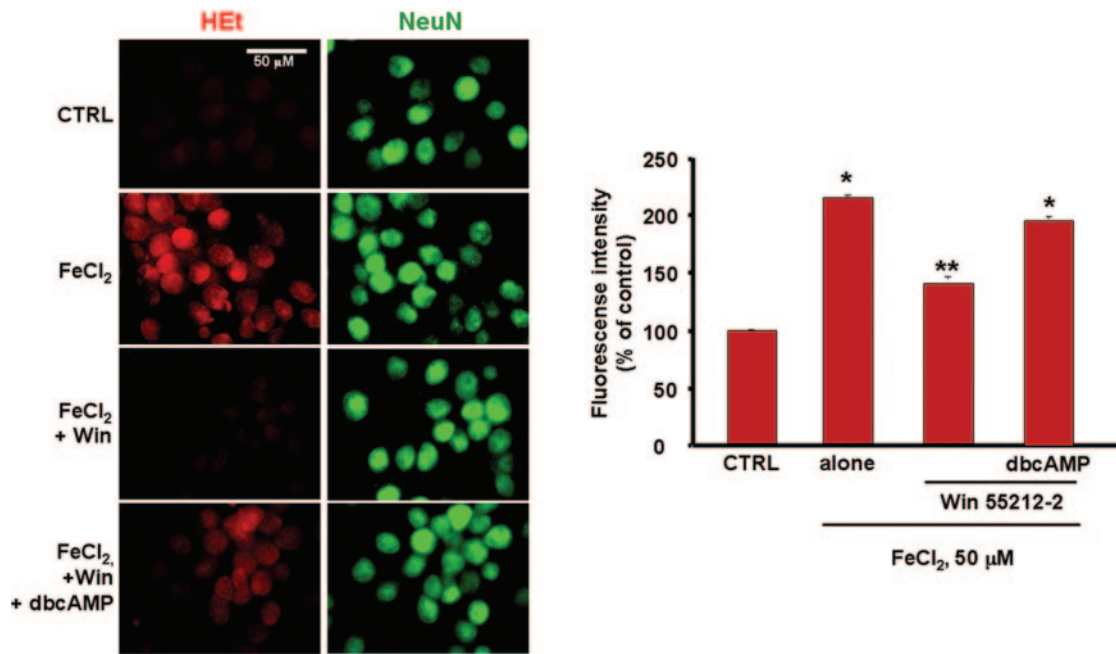


Fig. 6. Effects of Win55212-2 and dbcAMP on FeCl₂-stimulated production of reactive oxidative species. Cortical neuron cultures were exposed to 50 μM FeCl₂ for 24 h, in the absence and presence of 100 nM Win 55212-2 or Win 55212-2 plus 100 μM dbcAMP. Reactive oxidative species were measured by (red) nuclear fluorescence in cultures treated with hydroethidine (HET), and neuronal phenotype was confirmed by NeuN immunocytochemistry (green). Images at left are representative fields and data at right are means ± S.E.M. from 16 wells per condition. *, $P < 0.05$ compared with untreated cultures (CTRL); **, $P < 0.05$ compared with both untreated cultures and FeCl₂ alone (ANOVA and Student-Newman-Keuls tests).

NMDA-induced, peroxynitrite-mediated ganglion cell death was inhibited by cannabinoids, ~60% of the cannabinoid effect could be blocked by the CB1R antagonist SR141716A (El-Remessy et al., 2003), consistent with CB1R involvement. Our results are in closest agreement with this latter study.

One of the best characterized cannabinoid signaling pathways involves CB1R coupling to pertussis toxin-sensitive G_{i/o} proteins, producing inhibition of adenylyl cyclase and reduced production of cAMP (Childers and Deadwyler, 1996); however, the possible involvement of this system in the neuroprotective effects of cannabinoid has received little attention. Hampson and Grimaldi (2001) found that in cortical neuron cultures, cannabinoids prevented NMDA toxicity, but only in the presence of added cAMP. This contrasts with our finding that dbcAMP reversed the protective effect of Win 55212-2. Although not studying toxicity per se, Huang et al. (2002) obtained evidence for cAMP- and protein kinase A-mediated regulation of presynaptic CB1Rs, leading to disinhibition of glutamate release. Under excitotoxic conditions, this relationship might cause cAMP and protein kinase A to abrogate a protective effect of cannabinoids that involved suppression of glutamate release, although the role of such suppression in cannabinoid neuroprotection is uncertain. Consistent with our findings, this would suggest that cannabinoid-induced neuroprotection depends on reduction of cAMP-based signaling, although it would place the cAMP effect upstream, rather than downstream, of the receptor. The discrepancy may be more apparent than real, however, because Huang et al. (2002) noted they could not rule out a physiological action of protein kinase A downstream of the CB1R.

The finding that Win 55212-2 decreased and dbcAMP restored FeCl₂-induced ROS generation indicates that CB1R-induced, protein kinase A-sensitive neuroprotection is likely

to involve reduced ROS production. Protein kinase A-dependent production of ROS has been described in several systems, including leptin-stimulated endothelial cells (Yamagishi et al., 2001), tumor necrosis factor-treated fibrosarcoma cells (Van Herreweghe et al., 2002), and cardiomyocytes after hypoxia and reoxygenation (El Jamali et al., 2004). In a neuroepithelial tumor cell line, ROS production was enhanced by a signaling pathway that involved cAMP, protein kinase A, and the protein kinase A substrate cAMP response element-binding protein (Boissel et al., 2004). These precedents will be helpful in guiding future studies on mechanisms of cannabinoid neuroprotection.

References

- Bilak M, Wu L, Wang Q, Haughey N, Conant K, St Hillaire C, and Andreasson K (2004) PGE2 receptors rescue motor neurons in a model of amyotrophic lateral sclerosis. *Ann Neurol* **56**:240–248.
- Boissel JP, Bros M, Schrock A, Godtel-Armbrust U, and Forstermann U (2004) Cyclic AMP-mediated upregulation of the expression of neuronal NO synthase in human A673 neuroepithelioma cells results in a decrease in the level of bioactive NO production: analysis of the signaling mechanisms that are involved. *Biochemistry* **43**:7197–7206.
- Bouaboula M, Poinot-Chazel C, Bourrie B, Canat X, Calandra B, Rinaldi-Carmona M, Le Fur G, and Casellas P (1995) Activation of mitogen-activated protein kinases by stimulation of the central cannabinoid receptor CB1. *Biochem J* **312**:637–641.
- Chen Y and Buck J (2000) Cannabinoids protect cells from oxidative cell death: a receptor-independent mechanism. *J Pharmacol Exp Ther* **293**:807–812.
- Childers SR and Deadwyler SA (1996) Role of cyclic AMP in the actions of cannabinoid receptors. *Biochem Pharmacol* **52**:819–827.
- Davies SP, Reddy H, Caivano M, and Cohen P (2000) Specificity and mechanism of action of some commonly used protein kinase inhibitors. *Biochem J* **351**:95–105.
- Derkinderen P, Ledent C, Parmentier M, and Girault JA (2001) Cannabinoids activate p38 mitogen-activated protein kinases through CB1 receptors in hippocampus. *J Neurochem* **77**:957–960.
- Derkinderen P, Toutant M, Burgaya F, Le Bert M, Siciliano JC, de Franciscis V, Gelman M, and Girault J-A (1996) Regulation of a neuronal form of focal adhesion kinase by anandamide. *Science (Wash DC)* **273**:1719–1722.
- Derkinderen P, Valjent E, Toutant M, Corvol JC, Enslin H, Ledent C, Trzaskos J, Caboche J, and Girault JA (2003) Regulation of extracellular signal-regulated kinase by cannabinoids in hippocampus. *J Neurosci* **23**:2371–2382.
- El Jamali A, Freund C, Rechner C, Scheidereit C, Dietz R, and Bergmann MW (2004) Reoxygenation after severe hypoxia induces cardiomyocyte hypertrophy in vitro: activation of CREB downstream of GSK3beta. *FASEB J* **18**:1096–1098.

- El-Remessy AB, Khalil IE, Matragoon S, Abou-Mohamed G, Tsai NJ, Roon P, Caldwell RB, Caldwell RW, Green K, and Liou GI (2003) Neuroprotective effect of (-) delta9-tetrahydrocannabinol and cannabidiol in N-methyl-D-aspartate-induced retinal neurotoxicity: involvement of peroxynitrite. *Am J Pathol* **163**:1997–2008.
- Franklin A, Parmentier-Batteur S, Walter L, Greenberg DA, and Stella N (2003) Palmitoylethanolamide increases after focal cerebral ischemia and potentiates microglial cell motility. *J Neurosci* **23**:7767–7775.
- Galve-Roperh I, Rueda D, Gomez del Pulgar T, Velasco G, and Guzman M (2002) Mechanism of extracellular signal-regulated kinase activation by the CB1 cannabinoid receptor. *Mol Pharmacol* **62**:1385–1392.
- Gomez del Pulgar T, Velasco G, and Guzman M (2000) The CB1 cannabinoid receptor is coupled to the activation of protein kinase B/Akt. *Biochem J* **347**:369–373.
- Griffiths PD, Dobson BR, Jones GR, and Clarke DT (1999) Iron in the basal ganglia in Parkinson's disease. An in vitro study using extended X-ray absorption fine structure and cryo-electron microscopy. *Brain* **122**:667–673.
- Hajek M, Adamovicova M, Herynek V, Skoch A, Jiru F, Krepelova A, and Dezortova M (2004) MR relaxometry and 1H MR spectroscopy for the determination of iron and metabolite concentrations in PKAN patients. *Eur Radiol*, published online November 24.
- Hampson AJ and Grimaldi M (2001) Cannabinoid receptor activation and elevated cyclic AMP reduce glutamate neurotoxicity. *Eur J Neurosci* **13**:1529–1536.
- Hampson AJ, Grimaldi M, Axelrod J, and Wink D (1998) Cannabidiol and (-)-delta9-tetrahydrocannabinol are neuroprotective antioxidants. *Proc Natl Acad Sci USA* **95**:8268–8273.
- Herlitz S, Garcia DE, Mackie K, Hille B, Scheuer T, and Catterall WA (1996) Modulation of Ca²⁺ channels by G-protein $\beta\gamma$ subunits. *Nature (Lond)* **380**:258–262.
- Huang CC, Chen YL, Lo SW, and Hsu KS (2002) Activation of cAMP-dependent protein kinase suppresses the presynaptic cannabinoid inhibition of glutamatergic transmission at corticostriatal synapses. *Mol Pharmacol* **61**:578–585.
- Ikedo SR (1996) Voltage-dependent modulation of N-type calcium channels by G-protein $\beta\gamma$ subunits. *Nature (Lond)* **380**:255–258.
- Jin KL, Mao XO, Goldsmith PC, and Greenberg DA (2000) CB1 cannabinoid receptor induction in experimental stroke. *Ann Neurol* **48**:257–261.
- Liu R, Liu W, Doctrow SR, and Baudry M (2003) Iron toxicity in organotypic cultures of hippocampal slices: role of reactive oxygen species. *J Neurochem* **85**:492–502.
- Mackie K and Hille B (1992) Cannabinoids inhibit N-type calcium channels in neuroblastoma-glioma cells. *Proc Natl Acad Sci USA* **89**:3825–3829.
- Marsicano G, Moosmann B, Hermann H, Lutz B, and Behl C (2002) Neuroprotective properties of cannabinoids against oxidative stress: role of the cannabinoid receptor CB1. *J Neurochem* **80**:448–456.
- Moos T, Trinder D, and Morgan EH (2000) Cellular distribution of ferric iron, ferritin, transferrin and divalent metal transporter 1 (DMT1) in substantia nigra and basal ganglia of normal and beta2-microglobulin deficient mouse brain. *Cell Mol Biol (Noisy-le-grand)* **46**:549–561.
- Nagayama T, Sinor AD, Simon RP, Chen J, Graham SH, Jin K, and Greenberg DA (1999) Cannabinoids and neuroprotection in global and focal cerebral ischemia and in neuronal cultures. *J Neurosci* **19**:2987–2995.
- Noh JS and Gwag BJ (1997) Attenuation of oxidative neuronal necrosis by a dopamine D1 agonist in mouse cortical cell cultures. *Exp Neurol* **146**:604–608.
- Panikashvili D, Simeonidou C, Ben-Shabat S, Hanus L, Breuer A, Mechoulam R, and Shohami E (2001) An endogenous cannabinoid (2-AG) is neuroprotective after brain injury. *Nature (Lond)* **413**:527–531.
- Parmentier-Batteur S, Jin K, Mao XO, Xie L, and Greenberg DA (2002) Increased severity of stroke in CB1 cannabinoid receptor knock-out mice. *J Neurosci* **22**:9771–9775.
- Penn RB, Parent JL, Pronin AN, Panettieri RA Jr, and Benovic JL (1999) Pharmacological inhibition of protein kinases in intact cells: antagonism of beta adrenergic receptor ligand binding by H-89 reveals limitations of usefulness. *J Pharmacol Exp Ther* **288**:428–437.
- Rueda D, Galve-Roperh I, Haro A, and Guzman M (2000) The CB1 cannabinoid receptor is coupled to the activation of c-Jun N-terminal kinase. *Mol Pharmacol* **58**:814–820.
- Schaller B and Graf R (2004) Cerebral ischemia and reperfusion: the pathophysiological concept as a basis for clinical therapy. *J Cereb Blood Flow Metab* **24**:351–371.
- Tsao LI, Ladenheim B, Andrews AM, Chiueh CC, Cadet JL, and Su TP (1998) Delta opioid peptide [D-Ala2,D-leu5]enkephalin blocks the long-term loss of dopamine transporters induced by multiple administrations of methamphetamine: involvement of opioid receptors and reactive oxygen species. *J Pharmacol Exp Ther* **287**:322–331.
- Van Herreweghe F, Mao J, Chaplen FW, Grooten J, Gevaert K, Vandekerckhove J, and Vancompernelle K (2002) Tumor necrosis factor-induced modulation of glyoxalase I activities through phosphorylation by PKA results in cell death and is accompanied by the formation of a specific methylglyoxal-derived AGE. *Proc Natl Acad Sci USA* **99**:949–954.
- White BC, Sullivan JM, DeGracia DJ, O'Neil BJ, Neumar RW, Grossman LI, Rafols JA, and Krause GS (2000) Brain ischemia and reperfusion: molecular mechanisms of neuronal injury. *J Neurol Sci* **179**:1–33.
- Won SJ, Park EC, Ryu BR, Ko HW, Sohn S, Kwon HJ, and Gwag BJ (2000) NT-4/5 exacerbates free radical-induced neuronal necrosis in vitro and in vivo. *Neurobiol Dis* **7**:251–259.
- Yamagishi SI, Edelstein D, Du XL, Kaneda Y, Guzman M, and Brownlee M (2001) Leptin induces mitochondrial superoxide production and monocyte chemoattractant protein-1 expression in aortic endothelial cells by increasing fatty acid oxidation via protein kinase A. *J Biol Chem* **276**:25096–25100.
- Zecca L, Gallorini M, Schunemann V, Trautwein AX, Gerlach M, Riederer P, Vezzoni P, and Tampellini D (2001) Iron, neuromelanin and ferritin content in the substantia nigra of normal subjects at different ages: consequences for iron storage and neurodegenerative processes. *J Neurochem* **76**:1766–1773.
- Zecca L, Youdim MB, Riederer P, Connor JR, and Crichton RR (2004) Iron, brain ageing and neurodegenerative disorders. *Nat Rev Neurosci* **5**:863–873.

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