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# Drug-Induced Hypothermia Reduces Ischemic Damage

## Effects of the Cannabinoid HU-210

Ronen R. Leker, MD; Naomi Gai, BSc; Raphael Mechoulam, PhD; Haim Ovadia, PhD

**Background and Purpose**—Cannabinoids confer neuroprotection in several experimental paradigms, but the responsible mechanisms remain unknown. Therefore, we sought to examine whether the synthetic CB1 agonist HU-210 is capable of reducing ischemic damage and to determine the mechanisms responsible for such protection.

**Methods**—Sprague-Dawley rats underwent permanent middle cerebral artery occlusion (PMCAO). After dose-response and therapeutic time window-finding experiments, the rats were injected with HU-210 (45  $\mu\text{g}/\text{kg}$  IV) or vehicle 1 hour after PMCAO. Physiological parameters and cerebral blood flow in the peri-infarct zone were monitored. The animals were examined with a motor disability scale, and the infarct volumes were measured 72 hours later. We also examined the effects of the selective CB1 antagonist SR-141716 and of controlled warming on the neuroprotection conferred by HU-210.

**Results**—HU-210 reduced blood pressure and heart rate but did not alter the cerebral blood flow in the infarct border zone. Motor disability and infarct volumes were significantly reduced (by up to 77%;  $P < 0.05$ ) in animals treated with HU-210. A single injection of HU-210 significantly lowered the body temperature compared with vehicle as measured both at 1 hour ( $32.3 \pm 1.3^\circ\text{C}$  versus  $35 \pm 1.6^\circ\text{C}$ ;  $P = 0.0024$ ) and at 24 hours ( $31.5 \pm 2.5^\circ\text{C}$  versus  $37.25 \pm 0.3^\circ\text{C}$ ;  $P = 0.0031$ ) after PMCAO. The protective effects of HU-210 were partially reversed by pretreatment with SR-141716 but were completely abolished by warming of the animals to the levels observed in controls.

**Conclusions**—HU-210 confers robust protection against ischemic damage. This protection is mediated at least in part by binding to CB1 receptors and is also associated with the indirect protective effects of hypothermia. (*Stroke*. 2003;34:2000-2006.)

**Key Words:** cannabinoids ■ cerebral ischemia ■ hypothermia ■ neuroprotection ■ rats

Ischemic tissue damage is multifactorial and involves excitotoxicity,<sup>1</sup> reactive oxygen species,<sup>2</sup> inflammation,<sup>3</sup> and apoptosis.<sup>4</sup> Effective strategies to protect brain cells from these death-promoting mechanisms are lacking, and to date the only specific therapy for acute stroke remains the early use of thrombolysis to restore brain perfusion. This apparent inefficacy of neuroprotectants may be because most tested drugs were active against only 1 of the damaging processes associated with stroke, leaving the door open for other mechanisms to cause cellular death. One possible solution for this problem is to find drugs capable of neutralizing more than 1 damage-producing mechanism, but this search remains unsuccessful.

Cannabinoid CB1 receptors are widely distributed in the brain, and their number increases after brain ischemia.<sup>5</sup> CB1 agonists were shown to have a general inhibitory effect on neurons, potentially reducing the effects of damage-inducing mechanisms in ischemia.<sup>6-10</sup> Previous reports have shown that both endocannabinoids<sup>11</sup> and synthetic cannabinoids<sup>12,13</sup> may be protective against global and focal ischemic injury

but have not fully explored their mechanism of action. Furthermore, CB1 agonists have been implicated in causing hypothermia,<sup>14</sup> which is considered a powerful manipulation to induce neuroprotection in models of cerebral ischemia, hypoxia, and trauma.<sup>15,16</sup> Thus, CB1 agonists may be protective by direct effects mediated at the CB1 receptors and/or by indirect effects related to their hypothermic effects. The synthetic CB1 agonist HU-210 is much more potent than the endogenous CB1 ligands anandamide and 2-AG.<sup>17</sup> Therefore, we sought to test the effects of HU-210 in a model of focal cerebral ischemia.

## Materials and Methods

### Animals

Sprague-Dawley rats aged 13 weeks (weight, approximately 300 g) were used. The institutional animal care committee approved all experiments.

### Stroke Model

Animals were intubated and anesthetized with isoflurane 1.5%. The right femoral artery was cannulated for invasive monitoring of blood

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pressure and heart rate. Temperature and oxygen saturation were measured throughout the experiment and for 3 to 4 hours after drug administration with the use of external sensors. In these experiments body temperature was monitored but not controlled in both controls and active drug groups. Perfusion over the middle cerebral artery (MCA) territory was monitored throughout the experiment with a laser-Doppler probe. The proximal MCA was occluded as previously described<sup>18</sup> without removal of the occluding filament. This typically results in permanent MCA occlusion (PMCAO), yielding a large infarct involving cortical and subcortical zones.

### Dose-Response Experiment

We injected animals with vehicle or HU-210 at a dose range of 5 to 45  $\mu\text{g}/\text{kg}$  IV 1 hour after PMCAO ( $n=5$  to 8 per dose). Motor disability scores and infarct volumes were determined 72 hours after PMCAO, as detailed below.

### Therapeutic Time Window Assessment

We injected rats with vehicle or HU-210 45  $\mu\text{g}/\text{kg}$  IV 1, 2, 4, or 6 hours after PMCAO ( $n=5$  per group). Motor disability scores and infarct volumes were determined 72 hours after PMCAO, as detailed below.

### Effects of SR-171416

To examine the mechanism of action of HU-210, we administered the selective CB1 antagonist SR-141716<sup>17</sup> (10 or 20 mg/kg IV) 30 minutes before administration of HU-210 ( $n=10$ ) or vehicle ( $n=5$ ) after PMCAO. Animals underwent motor disability evaluation and assessment of infarct volumes 72 hours after PMCAO.

### Effects of Active Warming

We injected animals with HU-210 45  $\mu\text{g}/\text{kg}$  or vehicle 1 hour after PMCAO ( $n=5$  per group). We prevented the hypothermic effect of HU-210 by actively warming the animals with a heating lamp to the same temperatures observed in the vehicle group.

### Motor Disability Evaluation

All evaluations were performed blindly by 1 of the investigators unaware of the treatment regimen. Rats were weighed and examined daily with a standardized motor disability scale<sup>19</sup> with slight modifications. Animals were scored 1 point for each of the following parameters: flexion of the forelimb contralateral to the stroke when momentarily hung by the tail, extension of the contralateral hind limb when pulled from the table, and rotation to the paretic side against resistance. Additionally, 1 point was scored for circling motion to the paretic side when attempting to walk, 1 point was scored for failure to walk out of a circle 50 cm in diameter within 10 seconds, 2 points were scored for failure to leave the circle within 20 seconds, and 3 points were scored for inability to exit the circle within 60 seconds. Additionally, 1 point each was scored for inability of the animal to extend the paretic forepaw when pushed gently against the table from above, laterally, and sideways. Thus, an animal with a maximal deficit scored 10 points, and an animal with no deficit scored 0 points.

### Injury Size

Seventy-two hours or later after the surgery, the animals were reanesthetized and killed. Brain slices 2 mm thick were stained with 2,3,5-triphenyltetrazolium chloride (TTC) (2% solution) for 2 hours and preserved in 3.7% formaldehyde. Slices were photographed online with an image acquisition system. Estimation of the lesioned area was performed with the use of image analysis software. Infarct volumes are expressed as a percentage of the contralateral hemisphere.

### Psychotropic Effects

We evaluated psychomotor activity after injection of vehicle or HU-210 at all doses with a standardized open field activity test.<sup>20</sup> Animals were put into a large colored cage (50 $\times$ 30 cm) with a floor

### Physiological Variables in HU-210 and Vehicle-Treated Rats

	HU-210 45 $\mu\text{g}/\text{kg}$	Vehicle	<i>P</i> Values
<b>CBF</b>			
Filament insertion	15.8 $\pm$ 9%	15 $\pm$ 6.5%	0.95
3 hr after PMCAO	3.2 $\pm$ 0.75%	3.2 $\pm$ 1.4%	0.99
<b>Blood pressure</b>			
-60 min	102.4 $\pm$ 8.7	104.7 $\pm$ 14.4	0.89
60 min	84.5 $\pm$ 19.6	76.2 $\pm$ 9.1	0.71
90 min	59.5 $\pm$ 9.2	79.4 $\pm$ 9.6	0.17
120 min	53.5 $\pm$ 8.8	80.8 $\pm$ 8.3	0.05
180 min	46.2 $\pm$ 3	73.9 $\pm$ 6.6	0.005
<b>Heart rate</b>			
-60 min	234 $\pm$ 14	235 $\pm$ 14	0.6
60 min	212.5 $\pm$ 17.5	227 $\pm$ 2.5	0.48
90 min	187.5 $\pm$ 30.5	246 $\pm$ 4	0.19
120 min	167.5 $\pm$ 7.4	241 $\pm$ 19	0.07
180 min	178.5 $\pm$ 31.5	239 $\pm$ 21	0.26

CBF=cerebral blood flow measured by laser Doppler units. Data is presented as percentage of pre-occlusion flow  $\pm$ SD.

Time points for blood pressure and heart rate measurements are relative to drug administration.

divided into squares (3 $\times$ 3 cm). The number of squares stepped into with the use of both forefeet over 1 minute was logged. Tests were repeated for each animal at 30-minute intervals each hour for 5 hours on different days. We also tested the exploratory rearing tendency of the rats.<sup>21</sup> The rats were put into an unfamiliar regular cage, and the number of exploratory maneuvers in which the animal stands on its rear limbs and sniffs was measured over 1 minute. Tests were repeated for each animal at 30-minute intervals for 5 hours. After PMCAO, results for HU-210-treated animals were compared with those observed in vehicle controls and in naive animals.

### Statistical Analysis

Analysis was performed with the SigmaStat package (SPSS Inc). Data are presented as mean $\pm$ SD or mean $\pm$ SEM. Values were compared with the use of ANOVA with the Dunnett test and repeated-measures ANOVA.

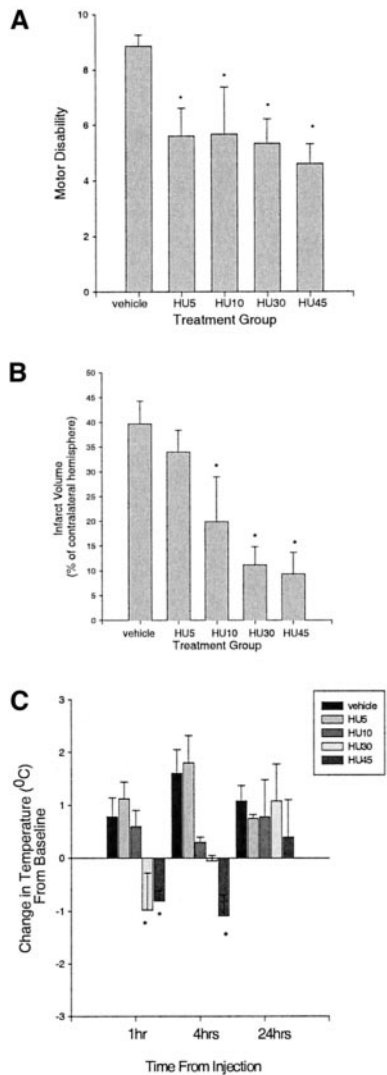
## Results

### Physiological Parameters

Physiological parameters are shown in the Table. HU-210 reduced systemic blood pressure and heart rate in a dose-dependent manner. However, cerebral blood flow in the ischemic zone was reduced to 15.8 $\pm$ 9% and 3.2 $\pm$ 0.75% of preischemic values immediately and 3 hours after filament insertion in the HU-210-treated group and 15 $\pm$ 6.5% and 3.2 $\pm$ 1.4% in vehicle-treated animals, respectively.

### Dose Response

HU-210 reduced motor dysfunction (Figure 1a) and infarct volumes (Figure 1b) in a dose-dependent manner. Maximal reductions in disability and infarct volumes were detected at the dose of 45  $\mu\text{g}/\text{kg}$ . At this dose, lesion volumes were reduced to 23% of their values in vehicle-treated rats. For the 10- and 30- $\mu\text{g}/\text{kg}$  doses of HU-210, infarct volumes were reduced to 33% and 28% of the volumes observed in vehicle controls, respectively. Administration of HU-210 at doses of 30 or 45  $\mu\text{g}/\text{kg}$  resulted in mild to moderate hypothermia

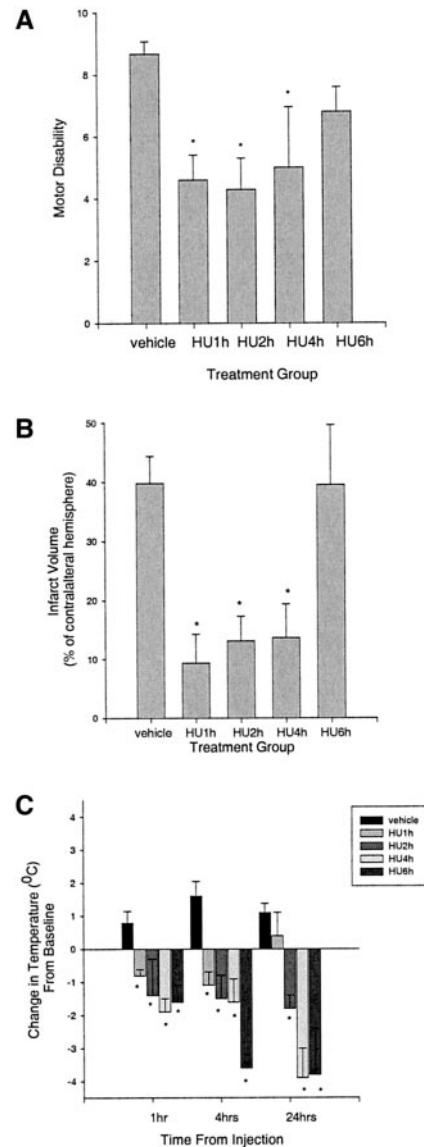


**Figure 1.** Dose-finding experiments. Animals were injected with vehicle or different doses of HU-210 (HU) 1 hour after PMCAO (n=8 per group). Motor disability (a) was assessed 72 hours after PMCAO, and infarct volumes were measured 72 hours after PMCAO (b). Temperature measurements were obtained at indicated time points after drug administration (c); data are presented as absolute change from baseline temperatures in degrees Celsius. Data are mean±SD. \*P<0.05 vs vehicle.

(Figure 1c). We chose the dose of 45 μg/kg because it leads to persistent mild hypothermia and consistent reductions in disability and infarct volumes despite significant lowering of systemic blood pressure. Individual inspection of TTC-stained brain slices from treated and untreated animals revealed that the major reduction in infarct volumes was due to sparing of the cortical penumbra.

### Therapeutic Time Window

Administration of HU-210 at 1, 2, or 4 but not 6 hours after PMCAO resulted in reduced motor disability (Figure 2a) and infarct volumes (Figure 2b) compared with vehicle controls. Infarcts were reduced to 23%, 33%, and 34% of the volumes observed in vehicle controls for administration of HU-210 at

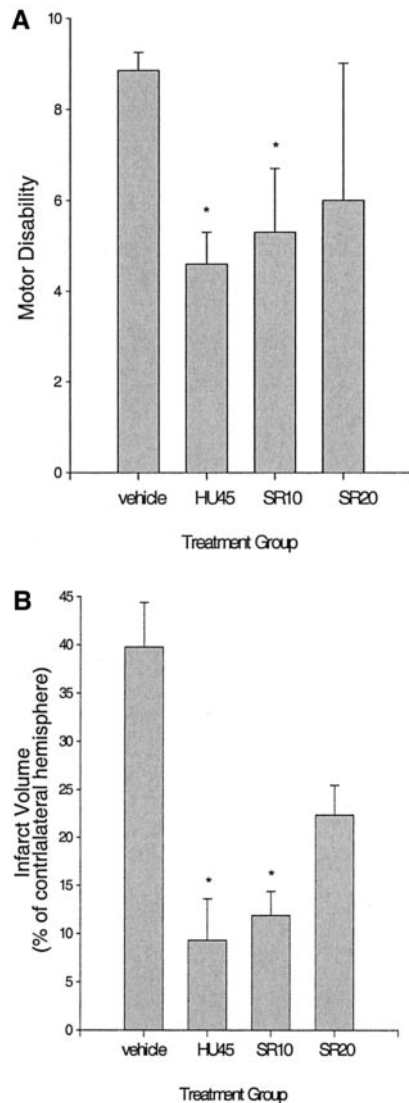


**Figure 2.** Therapeutic time window experiments. Animals were subjected to PMCAO and treated with vehicle or HU-210 45 μg/kg (HU) at different time points (n=8 per group). Motor disability was assessed 72 hours after PMCAO (a). The animals were killed 72 hours after PMCAO, and infarct volumes were measured (b). Temperature measurements were obtained at indicated time points after drug administration (c); data are presented as absolute change from baseline temperatures in degrees Celsius. Data are mean±SD. \*P<0.05 vs vehicle.

1, 2, and 4 hours, respectively. Thus, the therapeutic time window appears to extend to 4 hours after PMCAO. Of note, administration of HU-210 after 2, 4, or 6 hours resulted in a deeper and more consistent hypothermia than its administration at 1 hour after PMCAO, and this result did not vary between individual animals (Figure 2c).

### Effects of SR-141716

SR-141617 partially abrogated the protective effects of HU-210 on motor disability and infarct volumes (Figure 3a and 3b) in a dose-responsive manner. Thus, the antagonist led to

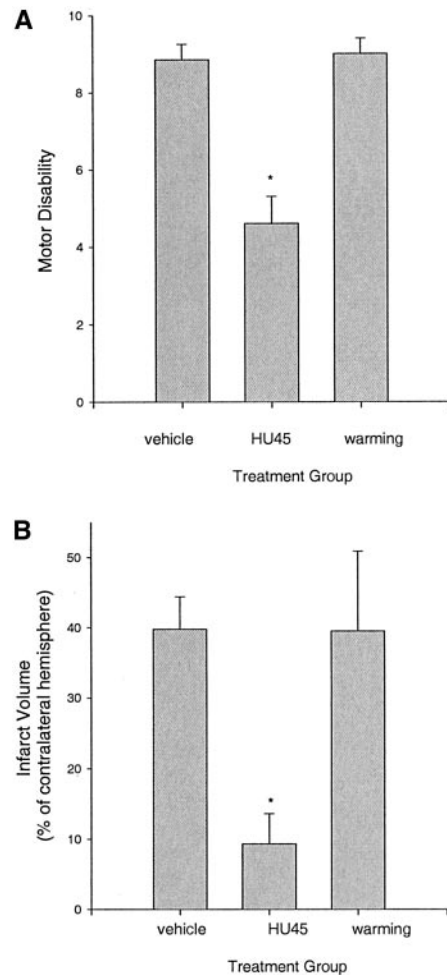


**Figure 3.** Effects of the cannabinoid CB1 receptor antagonist SR-141716. Rats were subjected to PMCAO and injected 1 hour afterward with vehicle, HU-210 (HU) 45  $\mu\text{g}/\text{kg}$ , or HU-210 45  $\mu\text{g}/\text{kg}$  in combination with the CB1 antagonist SR-141716 (SR) at different concentrations ( $n=7$  per group). Motor disability was assessed with a standardized scale 72 hours after PMCAO (a). The animals were killed 72 hours after PMCAO, and infarct volumes were measured (b). Data are mean  $\pm$  SD. \* $P<0.05$  vs vehicle.

infarct volumes that were 30% and 56% of the volumes observed in vehicle controls for the doses of 10 and 20 mg/kg, respectively. Administration of SR-141716 alone did not have any influence on motor disability or infarct volumes compared with vehicle. Importantly, high-dose SR-141716 also partially prevented the hypothermic effect of HU-210 but did not cause hyperthermia when administered alone. On pathological examination of the brain slices, a larger proportion of the cortical penumbral tissue was now incorporated into the infarct.

#### Effects of Active Warming

Warming of the animals to values observed in controls completely abolished the neuroprotective effects of HU-210



**Figure 4.** Effects of controlled rewarming. Rats were subjected to PMCAO and injected 1 hour afterward intravenously with vehicle ( $n=5$ ) or HU-210 (HU) 45  $\mu\text{g}/\text{kg}$  ( $n=10$ ). In 5 of the HU-210-injected animals, the temperature was actively controlled so that hypothermia was prevented with temperatures kept at values similar to those of vehicle controls for 24 hours. Motor disability was assessed 72 hours after PMCAO (a). The animals were killed 72 hours after PMCAO, and infarct volumes were measured (b). Data are mean  $\pm$  SD. \* $P<0.05$  vs vehicle.

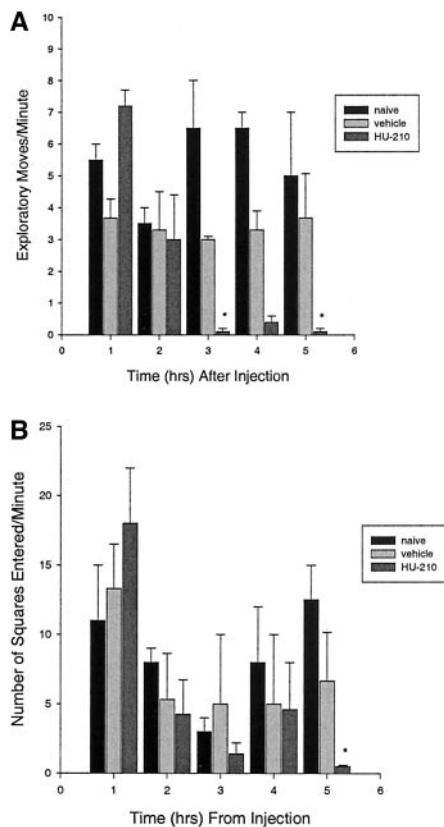
(Figure 4a and 4b). On detailed examination of the pathological data, the cortical zones that were protected with HU-210 were now infarcted.

#### Psychotropic Effects of HU-210

Animals treated with HU-210 demonstrated a significant reduction in motility as evaluated in the open field activity test in the first 72 hours after PMCAO (Figure 5a). Treated animals also performed significantly fewer exploratory motions than did control rats (Figure 5b). This reduction in activity implies psychotropism of HU-210.

#### Discussion

Administration of the CB1 agonist HU-210 after PMCAO markedly reduced motor disability and infarct volumes in a dose-dependent manner, with the best results obtained at a



**Figure 5.** Psychotropic effects of HU-210. Animals were injected with vehicle or HU-210 at a dose of 45  $\mu\text{g}/\text{kg}$  IV ( $n=7$  per group) 1 hour after PMCAO. Behavioral changes were measured over time with explorations in the rearing test (a) and square entrance in the open field behavior test (b) paradigms and also were compared with the results seen in naive animals ( $n=5$ ). Data are mean  $\pm$  SD. \* $P<0.05$  vs vehicle and naive animals.

dose of 45  $\mu\text{g}/\text{kg}$ . The reductions in lesion size and disability were still evident when the drug was given at 4 but not 6 hours after PMCAO. These findings corroborate previous reports on the neuroprotective effects of cannabinoids in ischemic brain injury.<sup>11–13</sup> The salutary effects of HU-210 were only partially abolished by the CB1 receptor antagonist SR-141716 despite the high dose used, suggesting that CB1-mediated mechanisms were responsible only in part for the neuroprotection. However, while we used higher doses of SR-141716 than those previously deemed to be sufficient for CB1 blocking,<sup>13,22</sup> we cannot rule out the possibility that using even higher doses would have completely prevented the protective effect of HU-210. Our findings are in accord with those of a recent study in head trauma in which the researchers needed to use higher than expected doses of SR-141716 to block the protective effect of the endogenous cannabinoid 2-AG,<sup>23</sup> suggesting that after brain injury it may be more difficult to block CB1 receptors. This could be due to the upregulation of endogenous cannabinoids under such circumstances, thereby increasing the competition at the CB1 site. Alternatively, the failure of SR-141716 to completely reverse the protective effects of HU-210 could be due to the higher affinity of HU-210 to the CB1 receptor, preventing the

antagonist from exerting its full effect. Furthermore, it is possible that the protection induced by HU-210 was mediated in part by concurrent mechanisms. Indeed, HU-210 administration was accompanied by moderate yet persistent hypothermia, which is suggested to be one of the mechanisms responsible for the neuroprotection because active warming of the rats completely abolished the protective effects. Therefore, the protective effects of HU-210 could be mediated by its binding to the CB-1 receptors and/or secondary to its production of hypothermia. Notably, HU-210 failed to protect the brain from ischemic damage at the dose of 5  $\mu\text{g}/\text{kg}$ , at which no hypothermia was observed, and the degree of protection was correlated with the depth of hypothermia observed at 4 hours after drug administration (Figure 1c). Thus, administration of HU-210 at a dose of 10  $\mu\text{g}/\text{kg}$  resulted in milder hypothermia and led to a smaller reduction in infarct volumes compared with higher doses that produced deeper hypothermia. Nevertheless, the fact that some degree of protection was observed at 10  $\mu\text{g}/\text{kg}$  suggests that hypothermia may not be responsible for the entire protective effect of HU-210.

As expected, HU-210 significantly lowered blood pressure and heart rate.<sup>24,25</sup> However, contrary to the findings of previous reports in normal rats,<sup>24</sup> it did not significantly alter cortical blood flow in the ischemic penumbra, eliminating local cerebral blood flow changes as potential avenues for neuronal protection or damage. Importantly, the protective effects of HU-210 were primarily evident in the cortical penumbra and were not observed in the ischemic subcortical core.

Endogenous cannabinoids present in the normal brain<sup>9,26</sup> are important in controlling cell fate and enhancing neuronal survival.<sup>27</sup> The mechanism of neuroprotection by cannabinoids involves downstream signal transduction related to G-protein coupling and reduced cAMP production, leading to reduced neurotransmitter release from presynaptic terminals.<sup>28</sup> The endocannabinoids 2-AG and anandamide as well as their CB1 receptors are induced after ischemia,<sup>5</sup> neurotrauma,<sup>23</sup> and other insults to the central nervous system.<sup>29</sup> CB1 agonists protect the brain against glutamate toxicity both by reducing its secretion from presynaptic terminals in a CB1-related mechanism and by blocking *N*-methyl-D-aspartate receptors in a CB1-independent mechanism.<sup>9,10</sup> Moreover, some reports suggest CB1 agonists to be antioxidants,<sup>7,8</sup> and still others found reduced production of proinflammatory cytokines after administration of CB1 agonists.<sup>30</sup> In addition, HU-210 induces hypothermia,<sup>14</sup> as also exemplified in the present experiment. This effect is probably mediated by direct binding of HU-210 to hypothalamic CB1 receptors.<sup>14</sup>

Physical hypothermia was shown to be neuroprotective in various experimental paradigms of stroke<sup>15,16,31,32</sup> by reducing many of the death-promoting mechanisms associated with cerebral ischemia.<sup>33–37</sup> Furthermore, the combination of putative cerebroprotective drugs with hypothermia shows synergistic effects.<sup>15</sup> Moreover, hypothermia may lead to conformational changes in existing peptides and also induces hypothermia-related genes, including RNA-binding proteins<sup>38</sup> that have a cold-shock domain that enables them to

bind denatured RNA and stabilize it, thus promoting cell survival.<sup>38</sup> In future experiments we propose to explore whether such proteins are induced after systemic administration of CB1 agonists.

Application of physical hypothermia is technically tedious and may result in serious adverse events.<sup>39</sup> Drug-induced hypothermia (eg, CB1 agonists, dopamine antagonists, and others) may serve as an alternative to physical hypothermia but depends on individual drug pharmacodynamics. Therefore, repeated dosing and timing schedules of administration of HU-210 must be explored to determine whether targeted prolonged hypothermia of 34°C could be achieved. Interestingly, the later HU-210 was injected after ischemic onset, the deeper and lengthier was the hypothermia achieved. The reason for this unexpected finding is currently unknown, but it may explain the fact that administration of the drug as late as 4 hours after ischemic onset still provided significant neuroprotection. Of note, no significant protection was observed when the drug was given 6 hours after stroke onset despite significant hypothermia. Conceivably, this may be explained by the minimal survival potential of penumbral tissue at this late stage.

One major issue of concern regarding the future use of cannabinoid agonists in stroke is that of safety. In the dose used in the present study, we found that HU-210 led to reduced locomotion and to hemodynamic and behavioral alterations in treated animals. These findings may be related to the general inhibitory activity of cannabinoids on cortical and subcortical motor, sensory, and autonomic systems. It is possible that use of this compound at lower doses that still bring about neuroprotection (eg, 10 to 30 µg/kg) may cause less psychotropic and hemodynamic side effects.

In conclusion, we found that the cannabinoid CB1 agonist HU-210 significantly reduced motor disability and infarct volume after focal irreversible cerebral ischemia. These salutary effects were probably mediated both by CB1-specific mechanisms and by its induction of hypothermia. However, this specific compound leads to significant behavioral alterations at the dose tested, precluding its use in humans. Nevertheless, this study shows that drug-induced hypothermia is protective against ischemic damage, and further experiments targeting other cannabinoid and noncannabinoid candidates appear to be indicated.

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