Carvacrol Together with TRPC1 Elimination Improve Functional Recovery after Traumatic Brain Injury in Mice

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Abstract

Death of Central Nervous System (CNS) neurons following traumatic brain injury (TBI) is a complex process arising from a combination of factors, many of which are still unknown. It has been found that inhibition of transient receptor potential (TRP) channels constitutes an effective strategy for preventing death of CNS neurons following TBI. TRP channels are classified into seven related subfamilies, most of which are Ca^{2+} permeable and involved in many cellular functions, including neuronal cell death. We hypothesized that TRP channels of the TRPC subfamily may be involved in post-TBI pathophysiology and that the compound 5-isopropyl-2-methylphenol (carvacrol), by inhibition of TRP channels, may exert neuroprotective effect after TBI. To test these suppositions, carvacrol was given to mice after TBI and its effect on their functional recovery was followed for several weeks. Our results show that neurological recovery after TBI was significantly enhanced by application of carvacrol. To better define the type of the specific channel involved, the effect of carvacrol on the extent and speed of recovery after TBI was compared among mice lacking TRPC1, TRPC3, or TRPC5, relative to wild type controls. We found that neurological recovery after TBI was significantly enhanced by combining carvacrol with TRPC1 elimination, but not by the absence of TRPC3 or TRPC5, showing a synergistic effect between carvacrol application and TRPC1 elimination. We conclude that TRPC1-sensitive mechanisms are involved in TBI pathology, and that inhibition of this channel by carvacrol enhances recovery and should be considered for further studies in animal models and humans.

Key words: head trauma; neuronal cell death; TBI

TRANSIENT RECEPTOR POTENTIAL (TRP) CHANNELS are essential components of biological sensors that detect environmental changes in response to a wide range of stimuli, such as temperature changes, mechanical force and mechanical injury, and changes in the chemical state of cells. They form an evolutionary conserved cation channel family consisting of seven subfamilies, which include nearly 30 human members.¹ The founding member of this family was discovered in *Drosophila* and was designated TRP.² TRP channels are classified into seven related subfamilies as described in detail in several reviews.^{1,3} TRP channels were shown to be involved in many cellular functions, including inflammation, cell adhesion, growth, differentiation, proliferation, death, and cell polarity.^{4–7}

Most members of this superfamily are Ca^{2+} permeable non-selective ion channels, and as such may be crucially involved in the pathological events following traumatic and ischemic brain injury.

There is increasing evidence suggesting the involvement of TRP channels in neurotoxicity. In particular, TRPM2 and TRPM7 from the TRPM channel subfamily are involved in neurotoxicity. Both channels are permeable to Ca^{2+} and activated by reactive oxygen species or oxygen-glucose deprivation, two hallmarks of brain injury, and these channels have been associated with cell death caused by anoxia,⁸ oxidative stress,⁹ and stroke.¹⁰ TRPM7 was shown to mediate anoxic neuronal cell death,¹¹ and it was reported that suppression of hippocampal TRPM7 prevented neuronal death following global cerebral ischemia.¹²

The mammalian canonical TRP channels (TRPC) are a subfamily of Ca^{2+} permeable nonselective cation channels, which are expressed in many tissues, including the brain. Members of this subfamily were found because of their sequence similarity to the *Drosophila* light-activated TRP channels. Overexpression of TRPC1 was shown to inhibit the activity of tumor necrosis factor

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(TNF)-induced transcription factor, NF- κ B,¹³ and to be involved in cell proliferation. Such cellular effect was shown to be associated with improved functional recovery after TBI.^{14,15} In contrast, a high level of apoptosis was observed in response to ischemic-reperfusion injury in cells overexpressing TRPC3,¹⁶ and TRPC1 was shown to be involved in glutamate mediated neurotoxicity.¹⁷

Sensitivity to a wide range of small molecules produced by plants is a characteristic feature of TRP channels. A large variety of natural products are known to modulate TRP channels of the TRPV, TRPM, and TRPA subfamilies.¹⁸ The compound 5-isopropyl-2-methylphenol (carvacrol), a major constituent of the essential oil of the popular culinary herb *Origanum vulgare* (oregano) shows a complex pharmacological profile. It activates the thermosensitive TRP channel, TRPV3 and TRPA1,¹⁹ and inhibits non-thermo TRP channels, the *Drosophila* TRPL (a member of the TRPC family) and mammalian TRPM7.²⁰ It is also anti-inflammatory by inhibiting cyclooxygenase (COX)-2 expression and activation of poly (ADP-ribose) polymerase (PARP).²¹ Even at high doses, carvacrol does not exert toxic effects,^{22,23} and, therefore, carvacrol is a natural compound with potential neuroprotective features.

We hypothesized that TRP channels of the TRPC subfamily may also be involved in pathophysiology after traumatic brain injury (TBI), and that carvacrol, by inhibition of TRP channels, may exert neuroprotective effects after TBI. To test these suppositions, carvacrol was given to mice after TBI. Mice were subjected to closed head injury (CHI) under isoflurane anesthesia, confirmed by loss of pupillary reflex, using a weight-drop device that falls over the left hemisphere, as previously described in detail.²⁴ Carvacrol (Sigma-Aldrich, St Louis MO) was dissolved in anhydrous ethanol:emulphor:saline (1:1:18) and injected intraperitoneally 1 h after induction of injury at 100 μ L per 10 g body weight.

One hour after CHI, the functional status of the mice was evaluated according to a set of 10 neurobehavioral tasks (neurological severity score [NSS]) that test reflexes, alertness, coordination, and motor abilities. One point was awarded for absence of reflex or failure to perform a particular task. Hence, a score of 10 reflected maximal neurological impairment. Immediately after evaluation of NSS at 1 h post-injury, mice were randomly assigned to vehicle or drug treatment, and NSS was evaluated again from 24 h up to 21 days. The effect of carvacrol treatment on functional recovery was followed for several weeks.

We first performed a dose-response study (0.5–25 mM, 75–3750 mg/kg) in Sabra mice (strain of the Hebrew University) and found that carvacrol at a dose of 75 mg/kg significantly improved the neurological outcome after CHI (see Fig. 1A) whereas higher doses were ineffective. Such a heterogeneous, bimodal dose-response curve is a typical behavior found for other natural products, either of plant or animal origin. Therefore, the cannabinoid (CB₁) receptor agonist Δ^9 -tetrahydrocannabinol exerted a biphasic control of fear coping strategies,²⁵ and when the endocannabinoid 2-arachidonoylglycerol was given to mice after CHI, only a dose of 5 but not 1 or 10 mg/kg significantly improved neurological outcome.²⁶

After establishing an effective dose, we tested the effect of carvacrol on C57BL/6 ($n=7-9$) mice lacking either TRPC1,²⁷ TRPC3,²⁸ or TRPC5, and compared the extent and speed of recovery after injury with that of their wild type (WT) C57BL/6 controls. Although TRPC5^{-/-} mice showed a trend toward better recovery than did WT mice (Fig. 1B), the absence of TRPC3 seemed to impair the recovery process and showed no recovery in certain motor tests. Interestingly, genetic elimination of TRPC1 in

FIG. 1. (A) Effect of different doses of carvacrol and vehicle on Sabra mice. Mice were subjected to closed head injury (CHI), 1 h later, neurological severity score (NSS) was evaluated and mice were treated with a single dose of carvacrol or vehicle. NSS was followed up to 21 days. * $p < 0.01$ when compared with vehicle-treated mice. (B) Effect of carvacrol on the recovery of TRPC^{-/-} mice after CHI. NSS of mice treated with carvacrol. Mice were subjected to CHI, 1 h later, NSS was evaluated and mice were treated with a single dose of 75 mg/kg carvacrol. NSS was followed up to 21 days. * $p < 0.005$ when compared with wild type (WT) mice. Error bars are standard error of the mean.

combination with carvacrol treatment resulted in a robust significant functional recovery (Fig. 1B). From 2 days after brain injury until the end of the experiment, carvacrol-treated TRPC1^{-/-} mice had highly significant (Mann-Whitney U test, $p < 0.005$) better (i.e., lower) NSS scores than did WT mice (day 2: 5.4 ± 0.2 vs. 6.2 ± 0.2 and day 21: 4.6 ± 0.3 vs. 5.7 ± 0.1).

One of the individual tests that comprise the NSS is walking on beams of different widths. Following carvacrol treatment, only TRPC1^{-/-} mice were able to regain the ability to walk on the 3 cm beam (only 12.5% failed this task from day 3 on), whereas 58% of the TRPC5^{-/-} mice and 93% of the TRPC3^{-/-} mice still failed to perform this task at the end of the 3rd week post-injury. In the WT

FIG. 2. Spontaneous recovery of wild type (WT) and TRPC1^{-/-} mice (no treatment with carvacrol), same experimental paradigm as in Figure 1, but without treatment.

group, the failure rate was 93% on day 3 and dropped to 71% by day 21. Interestingly, when testing the more difficult task, walking on a 1 cm wide beam, all WT, as well as all TRPC3^{-/-} and TRPC5^{-/-} mice, failed, whereas from 48 h post-injury, 40% of the TRPC1^{-/-} mice were able to perform the task.

In order to examine the possibility that the absence of TRPC1 is responsible for the improved recovery, we repeated the experiments with TRPC1^{-/-} and WT mice ($n=11$) in the absence of treatment, to evaluate spontaneous recovery. There was a non-significant trend toward better recovery in TRPC1^{-/-} mice (Fig. 2). Based on the experimental results, we conclude that carvacrol treatment and TRPC1 elimination have synergistic neuroprotective effects.

Our experiments identified additional ion channels involved in neurological recovery after head trauma, namely TRPC1 and TRPC3, which have opposing effects. TRPC3 seems to be important in the recovery of motor functions, whereas the absence of TRPC1 is not beneficial by itself, but becomes enhanced after carvacrol treatment.

The TRPC1 channel was shown to be involved in glutamate-mediated toxicity in hippocampal cultures.¹⁷ Previous studies have shown that TRP channels are involved in neurotoxicity after TBI. An additional study revealed that carvacrol is a potent antagonist of TRPM7, a channel that is activated under conditions of glucose-oxygen deprivation, probably by released reactive oxygen species. Moreover, it was shown that carvacrol is also a potent antagonist of the *Drosophila* TRPL channel, which belongs to the TRPC subfamily, whereas TRPC1, a member of this subfamily, was shown to participate in glutamate-mediated toxicity of hippocampal neurons. Together, the above mentioned findings suggest that treatment with carvacrol in addition to TRPC1 elimination might be neuroprotective after TBI.

In the present study, we first, as a proof of concept and dose-response study, found that carvacrol significantly improved functional recovery of Sabra mice at the lowest dose tested, whereas TRPC1 elimination had only a marginal effect on neurological recovery after CHI, as compared with WT controls. Strikingly, the combination of carvacrol application combined with TRPC1 elimination largely improved recovery after CHI, and this effect

was specific to TRPC1, and was not obtained by TRPC5 or TRPC3 elimination.

TRPC1 expressed in tissue culture cells does not produce a significant current in isolation. However, when expressed together with other signaling proteins/channels, a novel current appears (e.g., when co-expressed with TRPC5), suggesting that TRPC1 forms a heteromultimeric channel. Based on the results from the knockout animals, it is unlikely that carvacrol targets any of the tested TRPC channels. But surprisingly, the combination of carvacrol application and genetic elimination of TRPC1 exerts a synergistic neuroprotective effect. It is unlikely that carvacrol's effects are mediated by a single protein, but rather by multiple effectors, such as hypothermia induced by TRPV3 activation and protection from calcium overload by TRPM7 inhibition. As up-to-date interventions with a single pharmacological agent failed to show neuroprotective effects in human trials, our results point toward a strategy involving multiple targets. The TRPC channel usually forms heteromultimers; therefore, the absence of one TRPC protein can be compensated for by other TRPCs. Furthermore the pharmacological properties and biophysical properties are different depending upon the exact subunit composition. It is possible that by eliminating TRPC1 from a TRPC complex, the complex becomes more sensitive to carvacrol. Because of the complex biophysics of TRPC1, no specific pharmacological inhibitors were available to test this hypothesis.

Its polypharmacological profile makes carvacrol a promising compound for cytoprotective interventions. During the course of our study, an independent study was published. Carvacrol also showed protective effects in a middle cerebral artery occlusion mouse model. A dose similar (50 mg/kg) to the one used in our study reduced infarct volume and increased the level of phosphorylated Akt, an anti-apoptotic kinase.²⁹ Akt and TRPC1 regulate each other bidirectionally, whereas TRPC1 can be directly activated by Akt,³⁰ Akt activity is lower in TRPC1^{-/-} mice.³¹ Another possible mechanism for the polymodal action of carvacrol is not by directly modulating TRP channels, but by modulating associated proteins, such as Homer or stromal interaction molecule (STIM)1.³²

Our study demonstrates that both TRPC1 and TRPC3 are involved by opposing mechanisms in recovery after head trauma, such that whereas absence of TRPC3 prevents functional recovery, regardless of carvacrol treatment, elimination of TRPC1 is beneficial after treatment with carvacrol. The absence of TRPC5 does not seem to affect the intensity of or recovery from head trauma. In addition, we could show that carvacrol is a novel neuroprotective agent, the effects of which are potentiated by the absence of TRPC1.

Our results show that TRPC1 elimination in combination with carvacrol treatment is beneficial after CHI. Whereas direct pharmacological inhibition of TRPC1 is not a promising strategy, disrupting TRPC1 containing complexes with high-affinity peptides should be similar to genetic elimination of TRPC1. This strategy was successfully applied to prevent the neurotoxic effects of binding of NMDA-receptors to PDZ domains.³³

Carvacrol treatment in combination with disruption of TRPC1 complexes should be further explored in an animal model, with possible relatively rapid translation to the clinic, because of the safety profile of carvacrol.

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Author Disclosure Statement

No competing financial interests exist.

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