Possible Role of Spleen Derived Factors, Vanilloid Receptors and Calcitonin Gene-related Peptide in Diabetes Induced Hyperalgesia in Mice

Nadeem KHAN, Nirmal SINGH, and Amteshwar Singh JAGGI∗

Department of Pharmaceutical Sciences and Drug Research, Punjabi University, Patiala-147002, Punjab, India

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The present study was designed to investigate a possible role of vanilloid receptors, CGRP and spleen in the induction of diabetes induced hyperalgesia in mice. Tail flick latency, an index of hyperalgesia, was assessed using analgesiometer. Serum nitrite levels and an index of nitric oxide were analyzed using Griess reaction. Mice were rendered diabetic with streptozotocin (200 mg/kg -1, i.p.) and kept for 30 days for development of diabetic pain. To explore the involvement of spleen in diabetic pain, spleen homogenate supernatant (SHS) was prepared from spleen of 30th day diabetic mice and administered in normal mice for 14 days. Both in diabetic and SHS treated mice, significant degree of hyperalgesia was developed, suggesting the possible role of spleen derived factor in induction of diabetic pain. Moreover, the levels of nitric oxide were also elevated in 30 day diabetic mice and SHS treated mice. Administration of ruthenium red (1 mg/kg -1, i.p.), vanilloid receptor antagonist, and sumatriptan (50 mg/kg -1, i.p.), a CGRP release inhibitor, attenuated diabetes and SHS induced decrease in nociceptive threshold and increase in serum nitrite oxide levels. These results suggest that spleen derived factor induced activation of vanilloid receptors and CGRP release may be contributing in the development of hyperalgesia in diabetic mice.

Key words—diabetes; hyperalgesia; vanilloid receptor; calcitonin gene-related peptide (CGRP); spleen

INTRODUCTION

Diabetic patients frequently exhibit increased responsiveness to noxious stimuli (hyperalgesia) and hyper-responsiveness to normally innocuous stimuli (allodynia) that are often concurrent with a paradoxical loss of stimulus-evoked sensation.1,2 Similar to human diabetic pain, animal models such as streptozotocin (STZ) induced diabetic mice also demonstrate thermal hyperalgesia and mechanical allodynia.3,4

Transient receptor potential vanilloid (TRPV1) receptors are located on peripheral nerve fibers and known to function as a molecular integrator of different painful stimuli.5 Moreover, there have been reports indicating the involvement of TRPV1 receptors in development of inflammatory neuropathic pain.6 Further, up-regulation of TRPV1 receptors has been reported in the development of nerve injury induced neuropathic pain.7 The enhanced functioning of TRPV1 receptors in DRG neurons, probably due to increased phosphorylation, oligomerisation or reallocation of these receptors on the cell surface, may contribute to hyperalgesia in early stages of neuropathy.8

Stimulation of TRPV1 receptors is associated with release of neuropeptides including calcitonin gene-related peptide (CGRP) and induction of neurogenic inflammation.9 Intrathecal administration of CGRP has been associated with decrease in mechanical and thermal nociceptive threshold.10 Administration of a CGRP receptor antagonist has been reported to alleviate hyperalgesia in rodent model of neuropathy.11 The basal as well as capsaicin evoked CGRP release has been shown to increase by two times during diabetic state.12 Ruthenium red evoked CGRP release has been shown to increase by two times during diabetic state.13 Sumatriptan is 5-HT 1B/1D agonist and inhibits presynaptic release of CGRP.14

From our laboratory, it has been reported that spleenectomy restores the decrease in antinociceptive effect of morphine in diabetic mice, suggesting the interference of spleen factors with pain alteration during diabetes.15 In the light of above, the present study was designed to investigate the role of vanilloid receptors, CGRP and spleen in the pathogenesis of diabetic neuropathic pain.

MATERIALS AND METHODS

Swiss albino mice (20–30 g) of either sex were em-
ployed for the present study. Streptozotocin (Sigma Aldrich, U.S) was dissolved in 0.1 N citrate buffer (pH 4.5). Ruthenium red (Loba Chemie, Mumbai) and sumatriptan (Sun Pharmaceuticals, Ahmedabad) were dissolved in normal saline. The experimental protocol was approved by Institutional Animal Ethics Committee and care of the animals was carried out as per the guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Environment and Forest, Government of India (Reg. No.-107/1999/CPCSEA).

**Induction of Diabetes and Neuropathy in Mice**

Single dose of streptozotocin (STZ) (200 mg/kg, i.p.) was administered for induction of diabetes. Blood sample was obtained from retro-orbital sinus for glucose estimation. Mice with fasting blood glucose level of more than 14 mM L\(^{-1}\), on 16th day after STZ administration, were considered diabetic and were included for further study. The diabetic mice were kept for further 14 days for development of diabetic hyperalgesia.

**Estimation of Blood Glucose**

Blood glucose levels were estimated spectrophotometrically by glucose-oxidase method\(^{16}\) using a commercially available enzymatic kit.

**Measurement of Nociceptive Threshold**

Nociceptive threshold was determined by noting withdrawal latency in tail flick test using tail flick analgesiometer.\(^{17}\) The intensity of the radiant heat was adjusted to obtain a basal or pre treatment latency of 6–8 s in normal mice. Maximum cut off latency time was fixed at 10 s. Tail flick latency was expressed in seconds.

**Estimation of Serum Nitrite as an Index of Nitric Oxide Production**

Nitric oxide is rapidly oxidized to nitrite/nitrate and its half-life in biological system is very short.\(^{18}\) Therefore, the measurement of nitrite concentration is routinely used as an index of NO production.\(^{19,20}\) Serum nitrite levels were estimated by Griess reaction\(^{21}\) and values were expressed in \(\mu\)molL\(^{-1}\).

**Preparation of Spleen Homogenate**

After sacrificing the mouse by cervical dislocation, spleen was removed and immersed in 1% Minimal Essential Media (MEM, pH=7.8). The spleen was mashed, homogenized, and centrifuged at 3000 r.p.m for 10 minutes. Spleen homogenate supernatant (SHS) was used in place of mononuclear spleen cells to avoid any implication of immunogenic response.

**Experimental Design**

8 groups, each comprising 6 animals, were included in the present study.

1) Control group Group I (n=6): Mice were administered citrate buffer to serve as non-diabetic control animals. Tail flick latency was noted before and after administration of citrate buffer on different days i.e. 0, 1, 4, 10, 14, 17, 20, 24 and 30 day. Serum nitrite levels were noted before and after 30 day of buffer administration. Fasting glucose levels were monitored before and after administration of citrate buffer on 0, 7, 14, 21, 28 and 30 day.

2) Streptozotocin (STZ) treated group Group II (n=6): Mice were administered STZ (200 mg/kg, i.p.) to serve as diabetic control animals. Tail flick latency, serum nitrite and fasting glucose levels were noted on different days as described in group I.

3) Ruthenium red treated group Group III (n=6): Mice were administered ruthenium red (1 mg/kg\(^{-1}\), i.p.), daily for 14 days, starting after 16 days of STZ administration. Tail flick latency, serum nitrite and fasting glucose levels were noted on different days as described in group I.

4) Sumatriptan treated group Group IV (n=6): Mice were administered sumatriptan (50 mg/kg\(^{-1}\), i.p.), daily for 14 days, starting after 16 days of STZ administration. Tail flick latency, serum nitrite and fasting glucose levels were noted on different days as described in group I.

5) Groups treated with spleen homogenate supernatant (SHS) (a) Group V (n=6): Non-diabetic mice were administered normal saline solution 30 min prior to administration of SHS (1 ml/kg\(^{-1}\), daily) of non-diabetic mice for 14 days. Tail flick latency and blood glucose levels were noted on 0, 1, 2, 4, 8, 10 and 14 day of SHS administration. Serum nitrite levels were noted on 0 and 14 day after SHS administration.

(b) Group VI (n=6): Non-diabetic mice were administered normal saline solution 30 min prior to administration of SHS of 30 day diabetic mice for 14 days and mice were evaluated as described in group V.

(c) Group VII (n=6): Non-diabetic mice were administered ruthenium red (1 mg/kg\(^{-1}\), i.p.), 30 min prior to administration of SHS of diabetic mice, for 14 days and mice were evaluated as described in group V.
(d) Group VIII (n=6): Non-diabetic mice were administered sumatriptan (50 mg/kg, i.p.), 30 min prior to administration of SHS of diabetic mice, for 14 days and mice were evaluated as described in group V.

Statistical Analysis All the results were expressed as mean ± S.E.M. Two way analysis of variance (ANOVA) was followed by the Tukey’s multiple range test for post hoc analysis. The level of significance was fixed at p<0.05.

RESULTS

Effect of Pharmacological Agents on Blood Glucose Level Significant rise in glucose levels was noted in STZ treated mice as compared to citrate buffer treated mice. Neither ruthenium red nor sumatriptan modulated STZ induced rise in glucose levels. Administration of SHS of diabetic mice did not affect basal glucose levels. Moreover, ruthenium red and sumatriptan also did not modulate glucose levels in SHS treated mice (Table 1). Effect of Diabetes on Tail Flick Latency Time In streptozotocin rendered diabetic mice, there was significant decrease in tail flick latency time as compared to mice treated with citrate buffer. The increase in pain sensitivity reflects diabetes induced changes in non-myelinated nerve fibers, which transmit pain sensation of heat. This decrease in latency time was directly related to duration of diabetes and was more pronounced after 20 days of STZ administration (Fig. 1). Effect of Pharmacological Interventions on Tail Flick Latency Time in Diabetic Mice Administration of ruthenium red (1 mg/kg, i.p.) and sumatriptan (50 mg/kg, i.p.), attenuated diabetes induced decrease in tail flick latency time, noted on different days (Fig. 1).

Effect of Pharmacological Interventions on Serum Nitrite Level in Diabetic Mice Serum nitrite levels were increased significantly in diabetic mice as compared to normal non-diabetic mice. However, administration of ruthenium red and sumatriptan attenuated diabetes induced increase in serum nitrite levels in a significant manner (Fig. 2).

Effect of SHS on Tail Flick Latency Time and Serum Nitrite Levels Administration of SHS of non-diabetic mice did not produce any significant effect on tail flick latency time and serum nitrite levels in normal mice (Figs. 3 and 4). However, administration of SHS of 30th day diabetic mice significantly decreased tail flick latency time and increased serum nitrite levels in normal mice (Figs. 3 and 4).

Effect of Spleen Homogenate Supernatant (SHS) on Normal Mice Treated with Ruthenium Red and Sumatriptan Ruthenium red and sumatriptan significantly attenuated SHS of 30th day diabetic mice induced decrease in tail flick latency and increased serum nitrite levels (Figs. 3 and 4).

DISCUSSION

Streptozotocin (STZ) is well reported chemical agent to induce insulin dependent diabetes mellitus (IDDM) in mice. It is reported from our studies15,19 as well as from other laboratories20 that significant degree of hyperalgesia develops in mice after 4 weeks of STZ administration. Therefore, mice were kept for 4 weeks after STZ administration to provide sufficient time for hyperglycemia to affect pain perception.

### Table 1. Effect of Different Pharmacological Agents on Blood Glucose Levels (mg/dl)

<table>
<thead>
<tr>
<th>Groups</th>
<th>0th day</th>
<th>7th day</th>
<th>14th day</th>
<th>21st day</th>
<th>30th day</th>
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<tr>
<td>Control</td>
<td>87.8±10.3</td>
<td>82.4±18.4</td>
<td>92.4±16.9</td>
<td>94.8±9.4</td>
<td>98.2±10.2</td>
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<tr>
<td>Streptozotocin (STZ) treated</td>
<td>88.9±7.4</td>
<td>235.3±21.5*</td>
<td>252.7±28.4*</td>
<td>248.2±25.4*</td>
<td>245.0±20.1*</td>
</tr>
<tr>
<td>Ruthenium red treated</td>
<td>80.2±4.6</td>
<td>251.1±18.6*</td>
<td>248.3±20.4*</td>
<td>239.5±15.6*</td>
<td>261.9±16.1*</td>
</tr>
<tr>
<td>Sumatriptan treated</td>
<td>83.1±5.4</td>
<td>237.8±16.4*</td>
<td>258.7±15.7*</td>
<td>267.0±23.1*</td>
<td>238.3±19.7*</td>
</tr>
<tr>
<td>SHS (non-diabetic) control</td>
<td>80.9±4.9</td>
<td>236.8±10.5*</td>
<td>239.8±12.5*</td>
<td>256.0±21.8*</td>
<td>261.7±18.6*</td>
</tr>
<tr>
<td>SHS (diabetic) control</td>
<td>76.8±7.9</td>
<td>258.3±16.8*</td>
<td>251.9±18.3*</td>
<td>265.8±24.9*</td>
<td>239.7±17.9*</td>
</tr>
<tr>
<td>Ruthenium red treated SHS (diabetic)</td>
<td>90.1±12.1</td>
<td>230.7±18.2*</td>
<td>251.0±12.9*</td>
<td>262.9±17.9*</td>
<td>249.6±15.9*</td>
</tr>
<tr>
<td>Sumatriptan treated SHS (diabetic)</td>
<td>87.9±17.9</td>
<td>267.8±14.7*</td>
<td>245.9±12.5*</td>
<td>259.0±21.9*</td>
<td>240.7±16.9*</td>
</tr>
</tbody>
</table>

Each value is ±S.E.M. for six animals; *p<0.05 vs 0 day of each corresponding group.
Nitric oxide is rapidly oxidized to nitrite/nitrate and its half-life in biological system is very short. Therefore, the measurement of nitrite concentration is routinely used as an index of NO production.

In the present study, pain threshold was decreased significantly in STZ rendered diabetic mice. However, administration of ruthenium red, a TRPV1 receptor antagonist, and sumatriptan, a CGRP release inhibitor, resulted in attenuation of diabetes induced decrease in pain threshold. The noted anti-nociceptive actions of TRPV1 antagonist and CGRP release inhibitor indicate the key role of TRPV1 and CGRP in the induction of diabetic neuropathic pain. TRPV1 receptors are reported to be involved in induction of pain in tissue injury and inflammation. Moreover, these receptors are also reported to be up-regulated in nerve injury induced neuropathic pain. TRPV1 receptor antagonist, resiniferatoxin, has been demonstrated to prevent the development of hyperalgesia produced by ligation of sciatic nerve. CGRP plays an important role in pain signal transmission and CGRP antagonist has been reported to alleviate...
hyperalgesia in rodent model of neuropathic pain. Moreover, cizolirtine has been reported to attenuate diabetic neuropathic pain through presynaptic inhibition of CGRP release.27 Furthermore, activation of TRPV1 is associated with release of CGRP.24 So, it may be tentatively proposed that CGRP release triggered by TRPV1 activation is contributing in inducing hyperalgesia in diabetes.

In the present investigation, the serum nitrite levels were significantly elevated after 30 days of STZ administration. These results are in consonance with report from our laboratory19 and from others.28 In the previous study from our laboratory, it was reported that aminoguanidine, a nitric oxide synthase inhibitor, attenuated diabetes induced increased levels of nitric oxide along with decrease in tail flick latency.19 Serum nitrite levels are indicative of nitric oxide produced from eNOS (endothelium), not from nNOS synthase (neurons). So, the noted rise in serum nitrite levels in the present investigation suggests the enhanced expression of eNOS. Moreover, it has been documented that increase in nNOS is not responsible for diabetic neuropathy pain,29 while its decreased expression may do so.30 Further, it has also been noticed that net nitric oxide concentration is reduced during diabetes. Perhaps, this may be the reflective of differential effect of diabetes on expression of nNOS and eNOS. However, this paradoxical
effect of diabetes on nitric oxide needs further research. Administration of ruthenium red and sumatriptan attenuated diabetes induced increase in serum nitrite levels. It may be probably due to blockade of TRPV1 receptors and CGRP mediated nitric oxide release.31,32

In the present investigation, administration of spleen homogenate supernatant (SHS) of 30th day diabetic mice resulted in induction of hyperalgesia in normal non-diabetic mice. These results suggest that certain factors are released from spleen during diabetes, which contribute significantly in induction of hyperalgesia. However, administration of ruthenium red and sumatriptan attenuated SHS induced decrease in pain threshold. Although, the nature of spleen derived factors was not investigated, yet these results tentatively suggest that these factors activate TRPV1 receptors, release CGRP and induce hyperalgesia. Further, serum nitrite levels were also elevated as a consequence of SHS administration. Moreover, TRPV1 receptor antagonist and CGRP release inhibitor attenuated SHS induced elevated serum nitrite levels suggesting the inter-relationship of TRPV1, CGRP, spleen derived factor, nitric oxide and diabetic hyperalgesia. Nevertheless, further studies are needed for more conclusive evidence regarding this hypothesis by employing NO synthase inhibitor and capsaicin on SHS effect.

CONCLUSIONS

It may be concluded that spleen derived factors are contributing in the induction of diabetic hyperalgesia probably through the activation of vanilloid receptors, release of CGRP and nitric oxide. Nevertheless, further studies are required to elucidate the nature of spleen derived factor and inter-relationship between the contributing factors involved in induction of hyperalgesia due to diabetes.

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