INTRODUCTION
Among reactive compounds generated in diabetes mellitus is methylglyoxal (MG) (1-4). MG has proven to be an endogenous agonist of the transient receptor potential ankyrin 1 (TRPA1) (2, 5-7), a calcium-permeable cation channel expressed on a subpopulation of primary afferent nociceptive nerve fibers (8, 9, 11). In line with this, cutaneous administration of MG has pronociceptive effects in humans and wild-type mice, but not in TRPA1-/- mice (2, 4, 7, 11). Additionally, in the absence of hyperglycemia, systemic increase in MG levels induced by pharmacological inhibition or genetic deletion of glyoxalase 1, an enzyme detoxifying MG, has been shown to induce symptoms mimicking painful diabetic neuropathy in mice (7, 11, 12). Recently, various methylglyoxal scavengers (aminoguanidine, D-arginine and metformin) were shown to reduce the level of free methylglyoxal and attenuate mechanical hypersensitivity in diabetic rats (4). Together these findings suggest that methylglyoxal acting on TRPA1 channels exerts an important role in the development and maintenance of painful diabetic neuropathy. This hypothesis is further supported by the findings that pharmacological blocking of TRPA1 reduced pain hypersensitivity in the early phase of diabetes (13) and decreased the loss of nociceptive nerve endings and their function in the late phase of experimental diabetes (6). In a cohort of well-treated patients with short-term diabetes, however, a recent clinical study failed to find an association between the serum MG level and diabetic peripheral neuropathy (14).

Sustained TRPA1 activation and calcium influx are expected to produce changes in functional and even anatomical properties of nerve fibers (15). Earlier studies still leave open whether prolonged exposure to diabetes and the associated increase of endogenous MG modulate the pronociceptive effect of MG acting on TRPA1 on cutaneous nociceptor endings, the function of which is first influenced by diabetes (16). Therefore, we assessed whether pain behavior induced by intradermal administration of MG differs between animals with a streptozotocin (STZ) model of diabetes versus healthy controls.

Diabetes is associated with endoplasmic reticulum (ER) stress (17). Recently, it was shown that ER stress induced by intraplantar tunicamycin treatment in healthy controls produces a transient diabetic pain hypersensitivity-like condition that is restricted to the treatment site and that is not accompanied by blood glucose elevation (18). While TRPA1 has been shown to mediate pain hypersensitivity induced by diabetes (13, 19), it is still not known whether TRPA1 is involved in pain hypersensitivity induced by ER stress alone. Here, we assessed using pharmacological blocking of TRPA1 whether pain hypersensitivity induced by tunicamycin is...
driven by an endogenous TRP A1 agonist. Moreover, to further test the hypothesis that TRP A1 plays a similar role in ER stress- and diabetes-induced modulation of pain, we assessed whether these two conditions have similar effects on the pronociceptive effect induced by intradermal MG. To exclude the possibility that tunicamycin itself is a TRP A1 agonist which might explain the potential role of TRP A1 in the tunicamycin-induced ER stress, we performed an in vitro whole cell patch clamp study assessing whether or not tunicamycin activates TRP A1 channels.

MATERIALS AND METHODS

Experimental animals

The experiments were performed in male Hannover-Wistar rats (220 – 260 g; Harlan, Horst, The Netherlands) in Biomedical Helsinki. The experimental protocols were approved by the Experimental Animal Ethics Committee of the Regional Government of Southern Finland and the experiments were performed according to the guidelines of the European Communities Council Directive of 22 September 2010 (2010/63/EU). All efforts were made to limit distress, to use only the number of animals necessary to produce reliable scientific data, and to utilize alternatives to in vivo techniques, if available. The animals were housed in polycarbonate cages with a deep layer of saw dust, one to three animals in each cage, in a thermostatically controlled room at 24.0 ± 0.5°C. The room was artificially illuminated from 8.30 AM to 8.30 PM. The animals received commercial pelleted rat feed (CRM-P pellets, Special Diets Services, Witham, Essex, England) and tap water ad libitum.

Induction of diabetes mellitus

Diabetes mellitus was induced in one group of animals by intraperitoneal injection of streptozotocin (STZ; 50 – 60 mg/kg; Sigma-Aldrich, St.Louis, MO, USA) in citrate buffer (pH 4.5) as described elsewhere (20). The development of diabetes mellitus was confirmed at the end of experiments by measurements of blood glucose concentration (One Touch Ultra, Life Scan Inc., Milpitas, CA, USA). All STZ-treated animals developed diabetes and had a blood glucose level of > 20 mmol/l. If the animal had a weight decrease of > 20% or it showed signs of suffering, then the animal was immediately sacrificed by administering a lethal dose of pentobarbitone. All experiments in diabetic animals were performed 13 – 15 days after induction of diabetes.

Induction of endoplasmic reticular stress

ER (endoplasmic reticular) stress that is associated with a transient diabetic pain-like hypersensitivity condition, without accompanying elevation of the plasma glucose level, was induced in the skin of the hind paw of healthy control rats by injecting intraplantarly tunicamycin (20 µg/10 µl), an established inducer of ER stress (18, 21). The maximum mechanical hypersensitivity induced by intraplantar tunicamycin was obtained in 30 – 60 min and the maximum effect lasted at least for another 60 min. Therefore, testing of the MG-induced pronociception in the tunicamycin-pretreated skin or the attempt to reverse the tunicamycin-induced hypersensitivity by the TRP A1 antagonist did not start until 60 min after i.pl. administration of tunicamycin.

Assessment of mechanical pain sensitivity

Animals were habituated to pain testing procedures at least 30 min per day for two days before assessing drug effects on pain behavior. Since mechanical rather than heat hypersensitivity is a frequent problem in patients with peripheral neuropathy (16), the focus in testing of stimulus-evoked pain behavior was on mechanically evoked responses.

To assess mechanically evoked pain behavior, the frequency of withdrawal responses to the application of monofilaments (von Frey hairs) to the hind paw was examined as described earlier (22). A series of monofilaments (North Coast Medical, Inc., Morgan Hill, CA, USA) was applied in ascending order five times to the plantar skin at a frequency of 0.5 Hz. In one of the experimental conditions the testing was performed using forces varying from 1 g to 26 g, in one the experimental conditions forces varied from 0.4 g to 60 g, and in one condition a monofilament producing a force of 4 g was used, as described in the results section. A visible lifting of the stimulated hind limb was considered a withdrawal response. If the rat failed to withdraw to any of the five presentations of a monofilament, the response rate for the studied force level was 0%. If the rat withdrew every time the monofilament was applied to the paw, the response rate for the studied force level was 100%. Thus, an increase in the response rate represents facilitation of mechanical stimulus-evoked pain behavior (hypersensitivity).

Course of the study: methylglyoxal effect on pain behavior

The behavioral part of the study consisted of the following experimental conditions:

I) assessment of baseline response to mechanical stimulation in healthy controls (n = 6) and in diabetic animals (n = 6).

II) assessment of mechanical hypersensitivity induced by intradermal administration of MG in healthy controls (n = 6) and in diabetic animals (n = 6). MG was applied at two different doses (200 ng, 400 ng) to the plantar skin of the hind paw and the MG effect was assessed 10 min after its administration in this as in all other experiments. Saline was used for control injections. The order of testing different drug conditions varied between the animals, and the left and right limb were tested in consecutive order at 2 – 3 days intervals so that MG was not injected twice to the same limb.

III) Assessment of the MG-induced increase of the cumulative response rate to a series of monofilament in healthy control (n = 8) and diabetic (n = 12) animals. The drugs, drug doses and the time course of testing were identical to those in experiment II.

IV) Assessment of mechanical hypersensitivity induced by the second exposure to MG (400 ng) after MG at the same dose was applied one hour earlier either to the same site or an adjacent site in the plantar skin of healthy control animals (n = 6).

V) Assessment of mechanical hypersensitivity induced by tunicamycin and its attempted reversal with a TRP A1 antagonist in healthy control animals (n = 6). Monofilament test was performed before and at various time points after intraplantar administration of tunicamycin. One hour after tunicamycin administration the animals were administered intraplantarly the TRP A1 antagonist or DMSO (dimethylsulphoxide used to dissolve the TRP A1 antagonist). The rationale for administering tunicamycin 60 min before the TRP A1 antagonist was to have the maximum effect of tunicamycin overlap the maximum effect of the currently used TRP A1 antagonist, as indicated by previous results (18, 23). The two conditions (tunicamycin followed by TRP A1 antagonist or DMSO) were studied at two day intervals in counterbalanced order in two different limbs.

VI) Assessment of mechanical hypersensitivity induced by the high MG dose in skin pretreated 60 min earlier with tunicamycin or in a control skin area of healthy animals (n = 6). The order of testing different conditions and different limbs was varied and the testing interval was at least two days. In experimental conditions i and ii, the same animals were studied,
whereas in each of the other conditions separate groups of animals were used.

Animals were sacrificed with an overdose of pentobarbital after completion of the experiments.

**Cell culture for in vitro patch clamp study**

Human (HEK-Lac-t-rpA1 clone B22) and TRPA1-inducible HEK-293 cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with newborn-calf serum (10%), 25 mM N-2-hydroxyethylpiperazine-N’-2-ethane (HEPES), 2 mM L-glutamine, 1 mM Na-pyruvate, 100 U mL⁻¹ streptomycin and 20 µg mL⁻¹ hygromycin. In addition human TRPA1 growth media was supplemented with 0.3 mg and rat TRPA1 growth media with 0.5 mg genetin mL⁻¹. Cells were subcultured twice weekly.

**In vitro patch clamp recordings**

Patch clamp recordings of TRPA1 currents using the perforated patch configuration (by amphotericin) were conducted at 36 ± 1°C with an Axopatch 200B amplifier (Molecular Devices, Sunnyvale, CA, USA). A coverslip with adhered hTRPA1-HEK cells was placed in the recording chamber and perfused with extracellular solution consisting of (in mM) NaCl 143, KCl 4, CaCl₂ 1.2, glucose 5, HEPES 10 (pH 7.4 with NaOH; osmolarity adjusted to 301 ± 3 mOsm). Patch pipettes were pulled from borosilicate glass capillaries (Harvard Apparatus, Kent, UK) and had tip resistances of 1.5 – 3.5 MΩ when filled with a solution consisting of (in mM): K-glucanote 122, KCl 30, MgCl₂ 1, HEPES 5 (pH 7.2 with KOH; osmolarity adjusted to 290 ± 3 mOsm). The final concentration of amphotericin B (solubilized in dimethylsulfoxide) in the pipette was 0.24 mg m⁻¹.

The data was filtered at 1 kHz and sampled at 10 kHz (Digidata 1322A, Molecular Devices), with data acquisition and analysis performed using pClamp 9.2 software (Molecular Devices). Capacitance and series resistance were compensated, the latter at ≥ 60%.

**Drugs and their administration**

Intradermal injections of MG and saline vehicle were performed at a volume of 20 µl, while intraplantar injections of tunicamycin (20 µg), Chembridge-5861528 (10 µg) or its vehicle (DMSO; 100%) were performed at a volume of 10 µl, while intraplantar injections of the latter at ≥ 30 µg does not influence mechanical threshold of the healthy control limb (24). Saline was used as a control for MG injections.

**Statistical analysis**

Data analysis was performed using chi-square test, one- or two-way analysis of variance (ANOVA), with a mixed design when appropriate, followed by Tukey’s test (comparisons among three or more groups), or Student’s t-test (comparisons between two groups). P < 0.05 (two-tailed) was considered to represent a significant difference. GraphPad Prism 6 software for Windows (GraphPad Software Inc., La Jolla, CA) was used for analyzing the data.

**RESULTS**

**Induction of mechanical hypersensitivity by methylglyoxal**

The baseline response rate to a series of monofilaments two weeks after induction of diabetes was significantly higher in the diabetic than control animals (main effect of diabetes: \( F_{1,20} = 5.14, P = 0.047 \)) (Fig. 1A). The baseline hypersensitivity in the currently used sample of diabetic animals was weak or only moderate as indicated by post hoc tests, according to which the difference in the response rates between healthy controls and diabetic animals failed to reach significance at any single force (Fig. 1A).

MG applied intradermally (i.d.) in the plantar skin (0, 200 or 400 ng at a volume of 20 µl) had a dose-related effect on the absolute response rate induced by monofilament stimulation at an innocuous force of 4 g (main effect of MG: \( F_{3,20} = 6.40, P = 0.007 \)), and this effect varied with the experimental group (interaction between MG treatment and experimental group: \( F_{2,36} = 11.00, P = 0.001 \)) (Fig. 1B). Post hoc tests indicated that MG induced an increase in the absolute response rate only at the higher MG dose and only in the control group. Moreover, hypersensitivity induced by the higher dose of MG was significantly stronger in healthy controls than in diabetic animals (Fig. 1B).

In a separate group of animals, the MG-induced mechanical hypersensitivity was assessed by determining the MG-induced increase in the cumulative response rate to a series of monofilaments producing forces varying from an innocuous force of 0.4 g to a noxious force of 60 g. When assessed as the cumulative response rate, MG (0, 200 or 400 ng) induced significant mechanical hypersensitivity (main effect of MG treatment: \( F_{2,36} = 11.0, P = 0.0002 \)) (Fig. 1C). This effect varied with the experimental group (interaction between MG treatment and experimental group: \( F_{2,36} = 11.7, P = 0.0001 \)). Mechanical hypersensitivity induced by MG treatment was significantly stronger in healthy controls than in diabetic animals (main effect of diabetes: \( F_{1,20} = 6.9, P = 0.017 \)) (Fig. 1C). Post hoc testing indicated that mechanical hypersensitivity, as revealed by an increase in the cumulative response rate, was significant only after administration of the higher dose of MG and only in healthy controls (Fig. 1C).

To assess whether prior exposure to MG attenuates hypersensitivity induced by MG in healthy controls and whether the attenuation is due to peripheral, central or both mechanisms, intradermal MG (400 ng) was administered twice at a 60 min interval either to the same site in the plantar skin or to two adjacent sites. Hypersensitivity induced by the second administration of MG at the same plantar site was significantly reduced when compared with hypersensitivity induced by the second administration of MG at an adjacent site (\( t_{1} = 2.7, P = 0.04 \) (Fig. 1D).

**Effect of methylglyoxal treatment in tunicamycin-treated skin of control animals**

Tunicamycin treatment of the skin induces ER stress that is associated with a transient diabetic pain hypersensitivity-like condition, without an accompanying elevation of the blood glucose level (18). Here we studied whether mechanical hypersensitivity induced by tunicamycin as that induced by diabetes in earlier studies is TRPA1 mediated (13, 19).

Moreover, we assessed whether hypersensitivity induced by MG is reduced following tunicamycin pretreatment as in diabetic animals of the present study.

Tunicamycin (20 µg/10 µl i.pl.) induced in the treated plantar skin mechanical hypersensitivity that reached its peak
within 30–60 min and continued at the same level at least for an additional 60 min (main effect of time after tunicamycin treatment: $F_{6,84} = 8.51$, $P < 0.0001$; Fig. 2A). Mechanical hypersensitivity induced by tunicamycin was reversed by i.pl. treatment with 10 µg of Chembridge-5861528 (interaction between time and treatment with Chembridge-5861528 versus DMSO vehicle: $F_{6,84} = 3.07$, $P = 0.009$; Fig. 2A). Chembridge-5861528 alone at the i.pl. dose of 10 µg had no effect on monofilament-induced responses in control skin area of healthy rats ($t = 0.0$; not shown).

The effect of pretreatment of the plantar skin with tunicamycin (20 µl, i.pl.) on mechanical hypersensitivity induced by MG was assessed in a separate group of animals. MG (200 ng, i.d.) had a significant effect on mechanically evoked withdrawal responses assessed 10 min after the MG/saline control treatment (main effect of MG treatment: $F_{1,10} = 34.1$, $P = 0.0002$; Fig. 2B), and the MG-induced effect varied depending on the pretreatment with tunicamycin (interaction between MG treatment and the pretreatment condition: $F_{1,10} = 27.26$, $P = 0.0004$; Fig. 2B). Post hoc tests indicated that in the tunicamycin-pretreated skin the response was significantly stronger than the corresponding control response in the skin area that was not pretreated with tunicamycin (Fig. 2B).

**Attempt to activate transient receptor potential ankyrin 1 by tunicamycin: an in vitro patch clamp study**

Tunicamycin at a concentration of 30 µM exhibited little or no acute activation of hTRPA1 in whole cell patch clamp...
recordings at –70mV (n = 3; Fig. 3). As evident from the inward current induced by the subsequent exposure to allylisothiocyanate TRPA1 was highly expressed in the studied cells (n = 3; Fig. 3).

**DISCUSSION**

**Pain behavior induced by intradermal methylglyoxal in control and diabetic animals**

In healthy controls, intradermal administration of methylglyoxal (MG) produced mechanical hypersensitivity, which finding is in line with earlier results showing pronociceptive effects induced by cutaneous administration of MG (2, 4, 7) or other TRPA1 channel agonists (25-28). While there is accumulating evidence indicating that an increase of the endogenous MG level contributes to the diabetes-associated pain and hypersensitivity ((11), for reviews see (29), (30); however, (14)), the present behavioral results indicate that mechanical hypersensitivity induced by intradermal administration of MG was reduced in diabetes. Among possible explanations for this apparent paradox is that due to increased levels of endogenous TRPA1 agonists, such as MG, saturation of TRPA1-mediated transduction mechanisms in nociceptive nerve endings takes place in diabetes. This could explain the apparent paradox that diabetic animals can have a TRPA1-mediated pain hypersensitivity condition (6, 13, 19) but no further pronociceptive effect by intradermal injection of MG, as observed in the present study.

It was expected that if peripheral mechanisms have a key role in the reduction of hypersensitivity induced by intradermal MG in animals with diabetes or ER stress, the magnitude of hypersensitivity induced by second exposure to MG should be significantly reduced when the second MG dose is administered in the same site but not when the second MG dose is
administered at an adjacent site. In healthy controls of the present study, the magnitude of hypersensitivity induced by the second dose of MG was significantly lower in the adjacent than the same plantar administration site. This finding suggests that peripheral mechanisms may explain the reduction in intradermal MG-induced hypersensitivity in animals with prior MG treatment or with exposure to an elevated level of endogenous MGs such as in diabetes.

Additionally, it is also possible that TRPA1-amplified transmission in the spinal dorsal horn (31) contributes to the reduction of the MG-induced pronociceptive effect in diabetes. It has been shown in animals with diabetic pain hypersensitivity that a low intrathecal dose of TRPA1 antagonist has a strong antihyperalgesic effect, while intraplantarly an order of magnitude higher dose of TRPA1 antagonist is needed to produce a weak antihyperalgesic effect (19). This earlier result suggests that the TRPA1-mediated amplification of transmission has a key role in pain hypersensitivity following diabetes-induced systemic increase of MG or other endogenous TRPA1 agonists. Thus, it may be hypothesized that even if peripheral TRPA1-mediated transduction were suppressed by sustained elevation of MG in diabetes, the central TRPA1-amplified pronociception might predominate and explain diabetic pain hypersensitivity that is maintained by systemic increase of MG.

On the other hand, there is also evidence indicating that increased spinal level of TRPA1 agonists can suppress nociceptive transmission (32). Therefore, we cannot exclude the possibility that a central increase of endogenous TRPA1 agonists induced by ER stress, with or without diabetes, caused a block or saturation of nociceptive transmission, and thereby contributed to the suppression of the intradermal MG-induced hypersensitivity.

**Suppression of methylglyoxal-induced pronociception in an endoplasmic reticulum stress-induced and TRPA1-mediated hypersensitivity condition**

Diabetes is known to induce ER stress (see for references 17), and pain hypersensitivity in animals with experimental diabetes has been reduced by an ER stress blocker (18). Moreover, ER stress induced by intraplantar tunicamycin treatment of healthy control animals has been shown to produce a transient and locally restricted mechanical hypersensitivity condition, without an accompanying increase of blood glucose (18). These findings suggest that ER stress is an important underlying mechanism in diabetic pain hypersensitivity (18). The present results extend the earlier ones by showing that the mechanical pain hypersensitivity induced by ER stress in the tunicamycin-treated skin was reversed by pharmacological blocking of cutaneous TRPA1 channels. In patch clamp recordings, tunicamycin failed to activate the TRPA1 channel which finding is in line with the proposal that the hypersensitivity in the tunicamycin-treated skin was caused by ER stress-related generation of endogenous TRPA1 agonists rather than a direct action of tunicamycin on the pronociceptive TRPA1 channel. Previously we showed that mechanical pain hypersensitivity in diabetic animals is reversed by pharmacological blocking of TRPA1 (13, 19). Together these findings give support to the hypothesis that independent whether ER stress is induced by diabetes or by other means, ER stress generates endogenous TRPA1 agonists that exert a key role in maintenance of diabetic pain hypersensitivity. Moreover, intradermal administration of a pronociceptive dose of MG failed to increase mechanical hypersensitivity in the tunicamycin-pretreated healthy controls as well as diabetic animals. This finding is in line with the proposal that ER stress is a key mechanism in diabetes-induced modulation of pain sensitivity (18).

In the peripheral tissue, tunicamycin may have induced ER stress-related generation of endogenous TRPA1 agonists due to action on various cell types among which are fibroblasts, keratinocytes and Schwann cells that all have endoplasmic reticulum. It is noteworthy that in the periphery TRPA1 is expressed not only on sensory neurons but also on keratinocytes (33-35) and fibroblasts (35). Interestingly, oligodendrocytes that are related to Schwann cells in the periphery express TRPA1 (36). Moreover, fibroblasts (37) and keratinocytes (38) have been shown to contribute to nociception through an interaction with nociceptive nerve endings. These findings raise the possibility that pain hypersensitivity induced by endogenous TRPA1 agonists generated in ER stress and diabetes may be due to an indirect activation of nociceptors by TRPA1 expressed on fibroblasts or keratinocytes, due to direct activation of TRPA1 on nociceptors, or both.

Interestingly, MG that is generated in diabetes may have a dual role in TRPA1-mediated diabetic pain hypersensitivity, since MG has a direct TRPA1-mediated pronociceptive action (2, 6, 7). In parallel, MG per se induces ER stress (39) that promotes mechanical pain hypersensitivity (18) that according to the present results is also TRPA1 mediated. Similar mechanisms might contribute to mechanical hypersensitivity that has been observed in isolated nerve-skin preparation under hyperglycemic condition but not in normoglycemia or in tissue isolated from healthy animals (40). Namely, it may be hypothesized that in isolated tissue in which ER stress is present, increased endogenous TRPA1 agonist production ensues under hyperglycemic conditions.

Among limitations of this study is that while intraplantar injections of MG, an endogenous TRPA1 agonist generated in diabetes, were used in testing changes in TRPA1-mediated pain hypersensitivity following development of diabetes or non-diabetic ER stress, the results do not allow concluding whether the observed changes were due to an increase of endogenous MG, an increase of other endogenous TRPA1 agonists generated in diabetes and ER stress such as 4-hydroxynonenal, or both.

**Other potential mechanisms involved in methylglyoxal-induced effects**

It has been shown that Na+/Ca++ contributes to MG-induced facilitation of nociceptive neuron firing and pain hyperalgesia (4, 11). Additionally, a recent study reported that the action of MG on hyperpolarization-activated cyclic nucleotide-gated (HCN) channels contributes to increase in pain hypersensitivity (41). However, the earlier finding that MG treatment failed to induce pain behavior in animals with knockout of the TRPA1 channel (7) indicates that Na+/Ca++ or HCN may not alone explain the MG-induced pain behavior.

Since melatonin has been shown to suppress mechanical allodynia (42), it remains to be studied whether melatonin might cause seasonal and circadian changes in the MG-induced mechanical hypersensitivity. In the present study, diabetic animals were not treated. Since diabetes treatment e.g. with metformine has been associated with metabolic changes such as an increase of triglyceride levels (43), it would of interest to study whether these changes involve MG and influence its pronociceptive action.

**Conclusions**

Tunicamycin-induced ER stress as well as diabetes mellitus in previous studies (13, 19) produces mechanical pain hypersensitivity that is reversed by a TRPA1 antagonist. An
exposure to either of these conditions is followed by an equal reduction in hypersensitivity induced by intradermal administration of MG, a TRPA1 agonist. Patch clamp recordings indicated that tunicamycin itself is not a TRPA1 agonist. A potential explanation for the reduced pronociceptive effect of intradermal MG in animals with diabetes and/or tunicamycin-induced ER stress, is increased generation of endogenous TRPA1 agonists causing saturation of TRP A1-mediated transduction mechanisms in nociceptive nerve fibers or other peripheral mechanisms as indicated by the finding in healthy controls that hypersensitivity induced by second intradermal MG dose was reduced in the treatment site but not adjacent to it.

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