A. B. Middlemas · S. Agthong · D. R. Tomlinson

Phosphorylation of c-Jun N-terminal kinase (JNK) in sensory neurones of diabetic rats, with possible effects on nerve conduction and neuropathic pain: prevention with an aldose reductase inhibitor

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Abstract *Aims/hypothesis:* This study was designed to determine whether diabetes in rats is associated with phosphorylation of c-Jun N-terminal kinase (JNK) and one of its transcription factors, c-Jun, in sensory neurones innervating the lower limb. We also sought to determine which neuronal phenotypes are affected and to examine the effect of aldose reductase inhibition on JNK and c-Jun phosphorylation. *Methods:* Diabetes was induced in rats using streptozotocin. Phosphorylation of JNK and c-Jun in lumbar dorsal root ganglia was determined by binding of phospho-specific antibodies using western blots and immunocytochemistry. Neuronal phenotypes were characterised by binding of N52 (an antibody that recognises the heavy neurofilament protein; for large-diameter mechanoreceptors) and of calcitonin gene-related peptide and the plant glycoprotein lectin IB4 (for nociceptors). The efficacy of the aldose reductase inhibitor fidarestat was determined by measuring polyol pathway metabolites in sciatic nerve, and functionally by measuring the conduction velocity of motor and sensory nerves. *Results:* In control rats, activated JNK and c-Jun were primarily associated with mechanoreceptors; in diabetes this was increased, but a greater increase was seen in nociceptors. Phosphorylation was prevented in all cells by fidarestat, which normalised polyol pathway metabolites as well as motor nerve and sensory nerve conduction velocity. *Conclusions/interpretation:* Fidarestat-sensitive phosphorylation of JNK and c-Jun occurs in fast-conduction mechanoreceptors—the same class of neurones that registers the changes in sensory nerve conduction velocity—and in nociceptors. This supports the notion that mitogen-activated protein kinase phosphorylation, via the polyol pathway, may convert the direct effects of raised glucose into impaired

nerve conduction and neuropathic pain. For proof of this we await the availability of specific JNK antagonists formulated for in vivo use.

Keywords Aldose reductase · c-Jun · Fidarestat · JNK · MAP kinase · Neuropathy · Rats

Abbreviations CGRP: calcitonin gene-related peptide · DRG: dorsal root ganglion · IR: immunoreactivity · JNK: c-Jun N-terminal kinase · IB4: glycoprotein lectin from *Griffonia simplicifolia* · MAP kinase: mitogen-activated protein kinase · MNCV: motor nerve conduction velocity · SNCV: sensory nerve conduction velocity · TRITC: tetra rhodamine-isothiocyanate

Introduction

Decreased nerve conduction velocity is characteristic of diabetic neuropathy, occurring in human diabetic patients [1] and in diabetic laboratory animals [2]. Since the earliest demonstration that aldose reductase inhibitors prevented slowing of motor nerve conduction velocity (MNCV) in rats with experimental diabetes [3], it has become a well-defined end-point for the evaluation of this class of drug. Conduction changes have also been the most responsive component of neuropathic diabetic defects in the clinic [4, 5]. In spite of this, there is no proven link between exaggerated flux through the sorbitol pathway and the molecular basis of the conduction velocity deficit or of the other features of neuropathy.

The Diabetes Control and Complications Trial focused attention on elevated glucose as a primary cause of diabetes complications [6], suggesting that excessive glucose initiates biochemical derangements that ultimately develop into complications. We have shown that all three families of mitogen-activated protein kinase (MAP kinase), i.e. extracellular signal-regulated kinase (ERK), p38 and c-Jun N-terminal kinase (JNK), are activated in peripheral nerves of diabetic rats and patients and in cultured neurones exposed to high glucose concentrations [7]. More recently

A. B. Middlemas · S. Agthong · D. R. Tomlinson (✉)
Faculty of Life Sciences, University of Manchester,
Stopford Building, Oxford Road,
Manchester M13 9PT, UK
e-mail: david.tomlinson@manchester.ac.uk
Tel.: +44-161-2755769
Fax: +44-161-2755767

we implicated the MAP kinase p38 as a de facto glucose transducer in causing early nerve conduction deficits in diabetic rats [8] and our study showed that phosphorylation of p38 MAP kinase was inhibited by aldose reductase inhibitors. Since p38 MAP kinase and JNK are activated by similar cellular stresses, the present study was designed to test the hypothesis that JNK phosphorylation in neurones is also a product of exaggerated flux through the polyol pathway. This would further contribute to alterations in neuronal phenotype downstream of raised glucose. Thus, we examined the phosphorylation of JNK by glucose in sensory neurones in vitro and in diabetic rats in vivo. We also examined the next step in the signalling cascade, phosphorylation of c-Jun, and we studied the effects of inhibition of the polyol pathway, using the aldose reductase inhibitor fidarestat.

Materials and methods

Animals and diabetes

All studies used male Wistar rats (Charles River, Margate, UK) with starting weights in the range of 280–320 g. All studies were covered by a Project Licence awarded by the UK Home Office and were approved by the Local Animal Ethics Committee. Diabetes was induced by i.p. injection of streptozotocin (Sigma-Aldrich, Gillingham, Dorset, UK) freshly dissolved in normal saline at a dose of 55 mg/kg, administered the morning after an overnight fast. Three days later glucose was measured by reflectance photometry in tail vein blood and any streptozotocin-treated rats with blood glucose less than 15 mmol/l were rejected from the study. Animals were then group-housed with full access to food and water for 12 weeks and treatments were given as defined below.

Treatments

Aldose reductase inhibition was achieved using fidarestat given by gavage at a dose of 2 mg kg⁻¹ day⁻¹. Treatment was begun immediately after confirmation of diabetes (day

3 after streptozotocin) and was maintained until the end of the protocol.

Tissues and measurements

At the end of the designated protocol, rats were anaesthetised (isoflurane in oxygen) and MNCV and sensory nerve conduction velocity (SNCV) were measured exactly as described elsewhere [9]. Animals were then designated for either biochemical studies or for immunocytochemistry. In the former case they were exsanguinated from the right ventricle under anaesthetic and tissues were dissected out and placed in tubes on dry ice for subsequent assay. For immunocytochemistry, tissues were perfused with fixative under anaesthetic and dorsal root ganglia (DRG) were removed for postfixation exactly as described elsewhere [8].

From non-perfused rats, one sciatic nerve was removed for measurement of glucose, fructose and sorbitol by capillary gas chromatography of trimethylsilyl derivatives exactly as described elsewhere [10]. The contralateral sciatic nerve and the L4 and L5 DRG were removed for SDS-PAGE and western blotting, again exactly as described elsewhere [7]. Blots were exposed to either antibodies directed at a non-phosphorylated epitope of JNK or c-Jun to measure total protein (both rabbit polyclonal; anti-JNK [Santa Cruz; from Autogen Bioclear, Calne, UK] at a dilution of 1:1,000, and anti-c-Jun [New England Biolabs, Hitchin, UK]) or phospho-specific antibodies to measure phosphorylation of JNK or c-Jun (anti-JNK-phos; mouse polyclonal antibody, dilution 1:200 [Santa Cruz], and anti-c-Jun-phos rabbit polyclonal antibody, dilution 1:1,000 [from New England Biolabs]). Secondary antibodies were either anti-mouse horseradish peroxidase (HRP)-linked (1:5,000) or anti-rabbit HRP-linked (1:5,000), both from Cell Signaling Technology, via New England Biolabs. Enhanced chemiluminescence (Lumiglo; Cell Signaling Technology, via New England Biolabs) was used to detect the signal from the blot. The bands in the X-ray film were scanned and quantitated using the Molecular Analysis image analysis software package (Version 1.5, Bio-Rad, Hercules, CA, USA). All gels com-

Table 1 Indicators of diabetes, polyol pathway metabolites and nerve conduction velocities

	Final body weight (g)	Final blood glucose (mmol/l)	Sciatic nerve content (nmol/mg dry weight)			Conduction velocity (m/s)	
			Glucose	Sorbitol	Fructose	Motor	Sensory
Controls (<i>n</i> =12)	527±19	4.9±0.5	7.2±0.5	0.5±0.1	4.1±0.2	58.6±1.3 ^b	62.1±2.4 ^b
Diabetic untreated (<i>n</i> =12)	349±10	23.6±1.4	33.7±2.1 ^a	8.6±0.9 ^a	15.8±0.9 ^a	44.3±1.2	49.5±1.9
Diabetic+fidarestat (<i>n</i> =11)	336±11	29.4±1.4	41.4±3.1 ^{a,b}	0.3±0.1 ^c	4.2±0.4 ^c	56.7±1.9 ^b	62.2±2.7 ^b

Data are mean±SEM

Body weight and blood glucose values were not tested

Analysis by one-way ANOVA with Scheffé's range test, levels of significance are denoted by superscripts: ^a*p*<0.001 vs controls; ^b*p*<0.001 vs untreated diabetics; ^c*p*<0.05 vs untreated diabetics

prised 20 lanes, so that on every blot there were at least six lanes from control samples (six rats). Thus, within each gel, each diabetic sample from diabetic rats (either untreated or treated) was normalised to the mean value for all controls in the same gel. That done, phospho-specific immunoreactivity (IR) for either JNK or c-Jun was normalised to total protein (non-phospho-specific IR) for the same tissue sample. This enabled us to estimate the phosphorylation of JNK or c-Jun independently of any changes in expression.

For immunocytochemistry, sections of L4 or L5 DRG were stained for JNK or c-Jun IR using exactly the same antibodies as those used for western blots (see above), but at different dilutions (primaries, phospho-JNK, 1:400; other three, 1:200). Secondary IR was revealed, for both JNK primaries, using anti-rabbit fluorescein isothiocyanate (FITC) conjugate (1:200) from Jackson ImmunoResearch Europe, Soham, UK. For both c-Jun primaries, secondary IR was revealed using an anti-rabbit biotin-(strept)avidin kit (both dilutions 1:200) from Vector Laboratories, Peterborough, UK. For calcitonin gene-related peptide (CGRP) IR, we used a sheep polyclonal antibody (Affiniti, Biomol International, Exeter, UK) at 1:1,000 dilution, with anti-sheep tetra-rhodamine-isothiocyanate (TRITC) (raised in donkey) at 1:200. The lectin IB4 (a glycoprotein from *Griffonia simplicifolia*) was biotin-labelled and used at 10 µg/ml, followed by ExtraAvidin-FITC conjugate at 1:200 (both from Sigma-Aldrich). Large-diameter cell bodies were stained with N52 anti-mouse (Sigma-Aldrich) at 1:1,000 and the staining was revealed using anti-mouse Texas red conjugate (raised in donkey; Affiniti) at 1:200.

The staining of both phospho-JNK and phospho-c-Jun (both anti-rabbit antibodies) in the same section was achieved as follows. Sections were blocked with 10% normal goat serum for 1 h then incubated with the first primary antibody (phospho-JNK) for 36 h at 4°C, washed 3×10 min with PBS and incubated with conjugated anti-rabbit FITC for 2 h, then washed again with PBS for 3×10 min. To double-label with a second primary antibody (phospho c-Jun anti-rabbit), avidin-biotin was applied and sections were blocked again with 10% normal goat serum for 1 h, incubated with the second primary antibody (phospho c-Jun) overnight at 4°C then washed with PBS for 3×10 min. The second secondary antibody was incubated (rabbit biotinylated at 1:200) for 45 min, washed for 3×10 min with PBS then incubated with avidin-TRITC 1:200 for 45 min and washed again for 3×10 min with PBS. Control sections were not exposed to either primary antibody but were treated with two secondary antibodies as described above.

Statistical analysis

Group means were compared using one-way ANOVA and, where the *F* ratio indicated, individual means were compared using Scheffé's range test. In all tests a *p* value of <0.05 was considered significant.

Results

State of diabetes and treatment effects

Diabetic rats lost weight and were hyperglycaemic (Table 1). Their sciatic nerves contained increased glucose and the polyol pathway metabolites, sorbitol and fructose, the accumulation of which was normalised by the aldose reductase inhibitor, fidarestat, without attenuation of the

Fig. 1 Neuronal phenotypes in rat DRG (control animals) and staining for JNK and c-Jun. Single staining for total JNK only (a) and for total c-Jun (e). Double staining for phospho-JNK (green) and CGRP (red; b), N52 (red; c), IB4 (red; d). Double staining for phospho-c-Jun (green) and CGRP (red; f), N52 (red; g), IB4 (red; h). Scale bar=25 µm

Fig. 2 Levels of phospho-JNK and phospho-c-Jun in DRG from diabetic rats. **a** Western blots (each lane contains extracts made from bilateral L4+L5 DRG from a single rat) showing no change in total JNK in diabetes, but an increase in phospho-JNK, which was prevented by fidaresat treatment. This was not accompanied by marked changes in phospho-c-Jun on western blots made from the same extracts. **b–f** Marked increases in nuclear staining for phospho-JNK (**c**) and phospho-c-Jun (**f**) in DRG from diabetic rats compared with controls (**b** and **e**, respectively) and fidaresat-treated diabetic rats (**d** and **g**, respectively). Phospho-JNK was also increased in the cytosol of neurone cell bodies in diabetes (**c**). Scale bar=25 μ m

Fig. 3 Fluorescence intensity (cytofluorimetric scanning) regressed against cell body diameter (average of shortest and longest diameters) for phospho-JNK (**a**, **b**) and phospho-c-Jun (**c**, **d**) in

DRG from control (**a**, **c**) and diabetic (**b**, **d**) rats. Each point represents a single cell body. $n=3$ rats for each scatterplot

Table 2 Phospho-JNK and phospho-c-Jun immunoreactivity in western blots from DRG and sciatic nerve

	DRG			Sciatic nerve	
	JNK 56/54 kDa	JNK 46 kDa	c-Jun	JNK 56/54 kDa	JNK 46 kDa
Controls (<i>n</i> =12)	1.00±0.21	1.00±0.24	1.00±0.09	1.00±0.23	1.00±0.18
Diabetic untreated (<i>n</i> =12)	3.23±0.27 ^a	1.59±0.20	1.22±0.09	3.26±0.73 ^a	3.30±0.71 ^a
Diabetic + fidarestat (<i>n</i> =11)	2.00±0.44 ^c	1.33±0.07	0.96±0.10	1.18±0.33 ^b	1.92±0.44 ^b

Data are mean±SEM

In each tissue sample, immunoreactivity to phospho-specific antibodies was normalised to the immunoreactivity for total protein for either JNK isoforms or c-Jun. In each blot, the mean value for control samples (at least *n*=6 per gel) was calculated, then each lane was normalised to that value

Analysis by one-way ANOVA with Scheffé's range test, levels of significance are denoted by superscripts: ^a*p*<0.01 vs controls; ^b*p*<0.01 vs untreated diabetics; ^c*p*<0.05 vs untreated diabetics

severity of diabetes (Table 1). Inhibition of aldose reductase was also associated with a rise in nerve glucose, presumably due to prevention of its removal by the polyol pathway. Diabetic rats also showed reduced motor and sensory nerve conduction velocities; these were normalised by fidarestat treatment (Table 1).

Phosphorylation of JNK and c-Jun

Phosphorylated JNK and c-Jun were distributed unevenly among the mixed neuronal phenotypes constituting the lumbar DRGs. Accordingly, double staining was used to relate JNK and c-Jun to each of the major phenotypes (Fig. 1). Large cell bodies of fast-conducting mechanoreceptors were characterised by staining with N52, an antibody that recognises the heavy neurofilament protein [11]. These neurones contained the highest levels of phospho-JNK and c-Jun in ganglia from non-diabetic rats. The DRG nociceptors in these rats (characterised by either CGRP or IB4 staining) contained relatively low levels of phospho-JNK, but the nuclear staining for c-Jun was marked in both phenotypes (Fig. 1).

In ganglia from diabetic rats there was a marked increase in staining intensity for phospho-JNK in both cytoplasm and nuclei of all cells (Fig. 2). Cytofluorimetric scanning

showed that this represented a more marked increase in intensity in the small cell bodies of nociceptive neurones (Fig. 3). A similar shift in staining was seen for phospho-c-Jun, but this was confined to nuclei (Figs 2 and 3). Treatment of diabetic rats with fidarestat normalised the staining pattern for both phospho-JNK and c-Jun (Fig. 2). In western blots of DRGs from untreated diabetic rats there were no significant alterations in the measured levels of total JNK or c-Jun, but there was increased phosphorylation of JNK in diabetes, as is indicated by the IR to phospho-specific antibodies (Fig. 2 and Table 2). The small and insignificant change in phospho-c-Jun was matched by a similar shift in total c-Jun. Protein fractionation revealed at least two isoforms of JNK: a lighter IR with an apparent molecular weight of 46 kDa and a heavier/retarded isoform. On higher resolution gels this can be split into two, with apparent molecular weights of 54 and 56 kDa. In this study we did not separate these two isoforms. The heavier isoforms of phospho-JNK were increased three-fold in DRG from diabetic rats (*p*<0.01; Fig. 1 and Table 2). The 46 kDa isoform was increased numerically, but this was not significant. Phosphorylation of the 54/56 kDa isoform was reduced significantly (*p*<0.05) by treatment with fidarestat, but was not normalised (Table 2). The treatment had no significant effect on the 46 kDa isoform in DRG (Fig. 1 and Table 2). In sciatic nerve there

Fig. 4 Levels of phospho-JNK in sciatic nerve. **a** Western blots (each lane contains extracts made from sciatic nerve from a single rat) showing no change in total JNK in diabetes, but an increase in phospho-JNK, which was prevented by fidarestat treatment. **b–d** Marked increases in axonal staining for phospho-JNK (**c**) from diabetic rats compared with controls (**b**) and fidarestat-treated diabetic rats (**d**). Insets (**b**, **c**): fields at four times greater magnification than the main micrographs; there are no fluorescent halos around the myelin profiles, indicating no staining in Schwann cells. Scale bar=25 µm

Fig. 5 Double staining for phospho-JNK and phospho-c-Jun in DRG from diabetic rats. Staining for phospho-c-Jun (a) and phospho-JNK (b) is shown alone and combined (c). A negative control, without exposure to either primary antibody, is also shown (d). Scale bar=25 μ m

was significant ($p < 0.01$) phosphorylation of both 46 and 54/56 kDa isoforms in diabetes and the phosphorylation of both was reduced ($p < 0.01$) by treatment with fidarestat (Fig. 4 and Table 2). Closer examination of sciatic nerve staining for phospho-JNK (see insets in Fig. 4) showed that in controls staining in Schwann cells was of similar intensity to that seen in axons. Diabetes increased axonal staining markedly, without noticeably affecting Schwann cell staining.

Double labelling showed that staining for phospho-JNK and phospho-c-Jun was coincident in cell bodies of ganglia from diabetic rats; in general those neurones with the highest levels of phospho-JNK also showed the highest levels of phospho-c-Jun staining (Fig. 5).

Immunocytochemistry of transverse sections of sciatic nerve confirmed the observation from western blots that c-Jun was not present in axons (results not shown). However, staining for phospho-JNK was markedly intensified in the axoplasm of myelinated neurones in sciatic nerves from diabetic rats. This was not seen in nerves from fidarestat-treated rats (Fig. 4). Examination at higher magnification (Fig. 4, insets) showed that Schwann cell staining for phospho-JNK was low or absent in nerves from control rats, matching the intensity seen in most axons. However, there was no increase in phospho-JNK staining in Schwann cells in diabetic rats, unlike the intensification in axoplasm.

Discussion

Reduced nerve conduction velocity is now a classical hallmark of experimental diabetic neuropathy, as is prevention of this deficit by treatment with an aldose reductase inhibitor. This, taken together with the normalisation of nerve sorbitol and fructose, allows the conclusion

that the treatment with fidarestat used here was biochemically and functionally effective. This is important in judging effects of the inhibitor on JNK and c-Jun.

In sciatic nerve phospho-JNK was present in the axoplasm of large-diameter myelinated fibres and, in the DRG, in the cytoplasm of large cell bodies. These localisations are mutually consistent. In a separate study we have seen retrograde axonal transport of activated JNK [12], though the methods used did not permit identification of the neuronal phenotype involved. Diabetes caused an enhancement of JNK phosphorylation in both locations in the present study and this fits with the increased retrograde transport in diabetic rats observed before [12]. Taken together, these findings are consistent with the hypothesis that JNK is activated at peripheral sites in large-diameter myelinated fibres in diabetes and the activated kinase is carried back to the cell body, where it influences transcription. The latter suggestion is reinforced by the phosphorylation by diabetes of nuclear phospho-c-Jun seen in large-diameter cell bodies, where the transcription factor is co-localised with increased levels of phospho-JNK. We have also shown increased levels of phospho-JNK in sural nerve biopsies from diabetic patients with neuropathy, which suggests that this phenomenon of retrograde signalling extends beyond diabetic rodents.

The next level of understanding of the role of this phenomenon in diabetic neuropathy requires the ability to inhibit phosphorylation with specific JNK antagonists in vivo and study the functional consequences. We were able to do this with p38 MAP kinase [8], but specific JNK inhibitors for in vivo use are not yet available. The observation that fidarestat inhibited phosphorylation of JNK is important and suggests mechanisms to explain the effects of exaggerated polyol pathway flux in diabetes, but the broad spectrum of effects of aldose reductase inhibitors

precludes identification of functional outcomes of JNK phosphorylation. It is interesting and important to note that there was no apparent increase in phospho-JNK staining in Schwann cells (Fig. 4). This is striking because of the inhibition of JNK phosphorylation by fidarestat, an inhibitor of an enzyme thought to be primarily in the Schwann cell and not the axon [13].

The distribution of phospho-JNK in DRG cell bodies of normal rats was selective for different neuronal phenotypes, large mechanoreceptors showing the most intense staining. This distribution was altered in ganglia from diabetic rats, large cell bodies showing a modest increase but small cell bodies showing the most marked increase (Fig. 3). The same was true for phospho-c-Jun. It is clear that in the pain syndromes associated with diabetic neuropathy, there is an alteration of the phenotype of primary nociceptors [14, 15]. In chronic neuropathic pain models without diabetes there is abundant evidence for phosphorylation of MAP kinases as an early event in the development of pain; it is assumed that these signalling systems translate the (usually mechanical) stimuli provoking the pain to a nociceptor phenotype associated with hypersensitivity [16, 17]. Thus, the observation reported here implicates phosphorylation of JNK and one of its target transcription factors in the development of pain and allodynia in diabetic rats. In this regard, JNK may act in concert with p38 MAP kinase [8]. This also suggests a mechanism by which aldose reductase inhibitors may act to prevent or reduce pain in diabetic neuropathy, as they undoubtedly do in animal models [18, 19] and possibly also in patients [5, 20, 21].

The second outcome of the selective distribution is that phosphorylation of JNK in diabetes may form an early step in changes in phenotype of the large-diameter cell bodies, giving rise to fast-conducting myelinated fibres. Given the prevention of nerve conduction deficits by aldose reductase inhibitors, in association with their prevention of phosphorylation of JNK in nuclei, it is tempting to suggest a potential involvement of JNK in conduction deficits. However, as stated above, this requires specific JNK antagonists for proof. The fact that an inhibitor of p38 prevented the development of conduction deficits [8] does not rule out the involvement of JNK. The two MAP kinases are known to act on some common transcription factors, and one candidate for this commonality is c-Jun [22]. This may explain the phosphorylation of c-Jun seen here in nociceptors that do not contain activated JNK—as stated above, these small cell bodies show enhanced phosphorylation of p38 MAP kinase in diabetes [8] and this may be the mechanism of phosphorylation of c-Jun in these neurones.

Our previous studies on neuronal JNK present us with an apparent paradox alongside the present study. As stated earlier, it is currently not possible to inhibit specifically JNK phosphorylation *in vivo*, but this can be done *in vitro*. When we exposed cultures of adult DRG neurones to JNK inhibitors, we found an increase in cell death, offering the possible interpretation that activation of JNK may be life-preserving under these conditions [23]. However, such

neurones have switched to a regenerative phenotype, which is always associated with JNK activation, possibly as an essential component of that phenotype [24]. In contrast, JNK is not normally activated in intact (i.e. not regenerating) neurones *in vivo*, so that increased phosphorylation by diabetes may contribute to a dysfunctional phenotype.

In conclusion, this study demonstrates phosphorylation and nuclear translocation of JNK and c-Jun in sensory neurones of diabetic rats, phenomena attenuated by the aldose reductase inhibitor fidarestat. Given the potential involvement of MAP kinase phosphorylation in neuronal, retinal and renal complications of diabetes [25], this interaction with the polyol pathway may have broader importance.

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Conflict of interest statement None of the authors have any conflicts of interest in connection with this work.

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