A vitamin D₃ derivative (CB1093) induces nerve growth factor and prevents neurotrophic deficits in streptozotocin-diabetic rats

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Abstract

Aims/hypothesis. Streptozotocin-diabetic rats show impaired neurotrophic support by deficient nerve growth factor (NGF) in muscle and skin. We, therefore, examined a novel agent (CB1093; 1(S),3(R)-Dihydroxy-20(R)-(1-ethoxy-5-ethyl-5-hydroxy-2-heptyl-1-yl)-9,10-seco-pregna-5(Z),7(E),10(19)-triene), which induces expression of endogenous nerve growth factor.

Methods. We gave CB1093 orally followed by measurements of mechanical nociception, nerve growth factor, neuropeptides (immunoassay) and nerve growth factor receptors (western blots).

Results. In non-diabetic rats CB1093 caused dose-dependent increases in nerve growth factor production (140 % in soleus muscle and 190 % in sciatic nerve) and a mechanical hyperalgesia in the foot. There was also increased sciatic nerve expression of neuronal NGF target gene products, substance P (16 %) and calcitonin gene-related peptide (CGRP; 52 %). Depletions of nerve growth factor, substance P and CGRP in sciatic nerves of diabetic rats were prevented

by CB1093, which also increased soleus nerve growth factor concentrations to 30 % over those seen in non-diabetic rats and increased its mRNA expression in skin. The CB1093 did not affect expression of nerve growth factor receptors (trkA and p75^{NTR}) in dorsal root ganglia in control or diabetic rats, though the p75^{NTR} expression was reduced by diabetes. The mechanical hyperalgesia seen in diabetic rats treated with vehicle was not exacerbated by CB1093.

Conclusion/interpretation. These findings show that in animal models of diabetes it is possible to prevent depletions of nerve growth factor and the products of its neuronal target genes by oral treatment of a highly potent inducer of NGF gene expression. Pain is a possible side-effect, though this was a function of dose and was manifest more in controls than in diabetic rats. [Diabetologia (1999) 42: 1308–1313]

Keywords CGRP, nerve conduction velocity, nerve growth factor, NGF receptors, neuropathy, nociception, p75^{NTR}, substance P, trkA, vitamin D₃.

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Abbreviations: 1,25-(OH)₂D₃, 1,25-Dihydroxy vitamin D₃; CGRP, calcitonin gene-related peptide; EDL, extensor digitorum longis; MNCV, motor nerve conduction velocity; NGF-LI, nerve growth factor-like immunoreactivity; NGF, nerve growth factor; p75^{NTR}, p75 neurotrophin receptor; SNCV, sensory nerve conduction velocity.

Deficiencies in nerve growth factor (NGF) expression have been reported in skin and muscle [1,2] in diabetic rats and in skin biopsy specimens of patients, with an association of this deficiency with neuropathy [3]. There are also deficits in expression of neuronal genes that form targets for NGF which correlate with the NGF deficits [4] and which are reduced by NGF treatment [5]. This latter observation gives rationale to treatment of patients with NGF to prevent biochemical and functional aspects of neuropathy in those fibres, whose phenotypes are maintained by NGF [6]. Such treatment, however, is not without problems.

Physiologically, neurotrophins are derived from appropriate innervated target cells and are, therefore, offered to the neurones at discrete sites and in critically low concentrations. Disseminated delivery resulting from systemic treatment can cause NGF to reach its neurones at inappropriate concentrations and with imperfect time profiles, possibly causing overexpression of the neuronal phenotype. This, together with unrealistically high concentrations at the injection site, could cause undesirable effects [7].

An alternative approach is treatment with drugs which stimulate expression of endogenous NGF, with the advantage that it is released for neuronal capture at the appropriate site. This does not preclude overexpression of both NGF and neuronal phenotype and the dose of an inducer will be critical. A second advantage of such an approach would be the production of an orally active agent – NGF requires systemic injection – with the capacity to either enter or be excluded from the brain, depending on drug and therapeutic indication. Such a pharmacological approach was first proposed in general terms almost a decade ago [8]. Since then many molecules have been shown to induce NGF *in vitro* [9]. One of the more potent examples is 1,25-dihydroxyvitamin D₃ (1,25-(OH)₂D₃) [10–12], the biologically active metabolite of vitamin D₃. This cannot translate to a usable effect *in vivo* because the labilisation of Ca²⁺, via stimulation of the vitamin D receptor in bone, causes lethal hypercalcaemia, though 1,25-(OH)₂D₃ will increase NGF mRNA expression in the brain at sub-lethal doses [13,14]. In recent years, several 1,25-(OH)₂D₃ analogues have been discovered which act as efficient inducers of NGF but are less potent at causing hypercalcaemia. One such analogue is calcipotriol (MC903), which is equipotent with 1,25-(OH)₂D₃ as a NGF inducer, but has a much less pronounced effect on Ca²⁺ metabolism *in vivo* [15]. We have studied another 1,25-(OH)₂D₃ analogue, CB1093 [1(S),3(R)-Dihydroxy-20(R)-(1-ethoxy-5-ethyl-5-hydroxy-2-heptyn-1-yl)-9,10-seco-pregna-5(-Z),7(E),10(19)-triene], which induces NGF synthesis *in vitro* [16]. This study determined whether treatment of diabetic rats with CB1093 could prevent the NGF deficit and its effects in diabetic rats.

Materials and methods

Animals and induction of diabetes. Male Wistar rats (Charles River, Manston, Kent, UK; 10 weeks old; initial weights 300–320 g) were divided into six groups at random and fasted overnight. Three groups of animals were made diabetic with a single intraperitoneal injection of streptozotocin (55 mg/kg; Sigma, London, UK); 3 days later all diabetic animals had blood glucose concentrations higher than 15 mmol/l. Diabetic and control animals were age-matched and maintained for 8 weeks. Two groups of control and two groups of diabetic animals were given CB1093 daily by gavage. Two doses of CB1093

were used, 0.3 µg/kg and 1 µg/kg. One group of control animals and one group of diabetic animals were treated daily with vehicle. All animals were treated for a total of 8 weeks. An interim measurement of nociceptive threshold was made 4 weeks after the start of CB1093 treatment. Measurement of motor nerve conduction velocity (MNCV) and sensory nerve conduction velocity (SNCV) were done 8 weeks after commencement of CB1093 treatment. All animal procedures followed guidelines laid down by the United Kingdom Home Office.

Tissue collection. At the end of the study rats were stunned and exsanguinated and the right sciatic nerve excised and adjacent segments used for peptide analysis and for determination of NGF-like immunoreactivity (NGF-LI). Right hindlimb muscles, soleus and extensor digitorum longus (EDL) were dissected out and weighed. Samples from both soleus and EDL muscles were taken for assay of NGF protein content by ELISA. The L₄ and L₅ dorsal root ganglia from one side were removed and processed for western blotting.

Nociceptive testing. All experiments were carried out with the identity of the rats masked from the investigator. All experiments were within guidelines and using procedures approved by the Home Office of the UK Government. The mechanical nociceptive threshold was quantified using an Analgesy-meter (Ugo Basile, Milan, Italy) exactly as described elsewhere [17]. Weekly training sessions were conducted for the first 4 weeks of the study before the definitive measurements were made at the end of week 4; this was done 24 h after treatment with CB1093.

Measurement of nerve conduction velocity. Motor and sensory nerve conduction velocities were measured from M wave latencies and H reflexes, under Hypnorm (0.6 ml/kg, intramuscular) and diazepam (2.5 mg/kg, i.p.) anaesthesia, exactly as described elsewhere [18].

Nerve growth factor and neuropeptide assays. The sciatic nerve segments were homogenised, extracted and assayed for NGF [5], substance P and CGRP as described elsewhere [19].

Measurement of NGF mRNA in foot skin. Total RNA isolated from homogenates (Polytron, Kinematica, Lucerne, Switzerland) of foot skin were homogenised as described previously [20]. Nerve growth factor mRNA was then measured relative to total RNA by northern blotting as described elsewhere [4].

Statistical analysis. All data are presented as the arithmetic means ± 1 SD. Statistical comparisons were made using two-way analysis of variance (ANOVA), with post-hoc range tests (Student-Newman-Keuls) for comparisons between the three diabetic or between the three control groups. Statistical significance was assessed at $p < 0.05$. All data underwent logarithmic transformations to maintain homogeneity of variance.

Results

Animal weights and diabetes. Diabetic rats had pronounced hyperglycaemia and progressive body weight loss in comparison with control animals (Table 1). Neither dose of CB1093 affected blood glucose, body weight or muscle weight in control or diabetic rats (Table 1).

Nociception. Diabetic rats treated with vehicle had a lower ($p < 0.05$) paw pressure threshold than controls

Table 1. The effects of CB1093 on body weights, plasma glucose and hindlimb muscle weights from control and diabetic rats

	Body weights (g)		Plasma glucose (mmol/l)	Skeletal muscle weights (mg)		
	Initial	Final		Soleus	EDL	Gastrocnemius
Vehicle-treated Control	295 ± 6	498 ± 26	9.5 ± 0.9	201 ± 27	822 ± 84	1257 ± 181
Control + 0.3 µg/kg CB1093	296 ± 16	490 ± 52	9.2 ± 0.6	200 ± 35	809 ± 101	1333 ± 190
Control + 1 µg/kg CB1093	289 ± 18	459 ± 34	8.9 ± 0.9	197 ± 26	760 ± 121	1348 ± 136
Vehicle-treated Diabetic	315 ± 12	310 ± 30	45.2 ± 2.4	125 ± 16	408 ± 134	644 ± 148
Diabetic + 0.3 µg/kg CB1093	302 ± 18	288 ± 41	50.8 ± 2.4	127 ± 24	420 ± 106	623 ± 185
Diabetic + 1 µg/kg CB1093	311 ± 6	282 ± 48	51.1 ± 2.4	129 ± 16	424 ± 74	624 ± 161

Data are means ± 1 SD ($n = 8-10$). There were no significant effects of CB1093 in either control or diabetic groups; massive differences between diabetic and non-diabetic rats were not tested

Table 2. The effects of CB1093 on paw pressure thresholds and nerve conduction velocities in control and diabetic rats

	Paw pressure threshold (g)	Nerve conduction velocity (m/s)	
		Motor	Sensory
Vehicle-treated Control	333 ± 30	46.3 ± 3.8	51.2 ± 7.8
Control + 0.3 µg/kg CB1093	253 ± 13 ^b	47.4 ± 4.4	47.6 ± 4.0
Control + 1 µg/kg CB1093	240 ± 13 ^b	51.9 ± 4.8	47.8 ± 4.2
Vehicle-treated Diabetic	193 ± 17 ^a	40.0 ± 3.5 ^a	41.4 ± 4.0 ^a
Diabetic + 0.3 µg/kg CB1093	180 ± 10 ^a	42.9 ± 3.7 ^a	44.5 ± 8.3
Diabetic + 1 µg/kg CB1093	167 ± 7 ^a	43.4 ± 6.7 ^a	41.5 ± 5.1 ^a

Data are means ± 1 SD ($n = 6$). ^a denotes $p < 0.05$ vs equivalent (i.e. same drug treatment) controls; ^b denotes $p < 0.05$ vs vehicle treated controls

Table 3. The effects of CB1093 on nerve growth factor-like immunoreactivity (NGF-LI), substance P-like immunoreactivity (SP-LI) and calcitonin gene related peptide-like immunoreactivity (CGRP-LI) in the sciatic nerve and the NGF-LI content of soleus and extensor digitorum longus (EDL) muscle of control and diabetic rats

	Sciatic nerve content			Skeletal muscle NGF-LI content	
	SP-LI ng/cm nerve	CGRP-LI pg/cm nerve	NGF pg/cm nerve	Soleus pg/mg tissue	EDL pg/mg tissue
Vehicle-treated Control	189 ± 49	4.8 ± 1.0	92 ± 25	0.99 ± 0.31	2.76 ± 0.89
Control + 0.3 µg/kg CB1093	184 ± 43	3.2 ± 0.4	127 ± 18	1.61 ± 0.67 ^c	2.37 ± 0.62
Control + 1 µg/kg CB1093	220 ± 35	7.3 ± 2.0 ^a	267 ± 134 ^c	2.37 ± 0.72 ^c	2.69 ± 0.18
Vehicle-treated Diabetic	96 ± 47 ^a	3.1 ± 0.9 ^a	40 ± 13 ^a	0.60 ± 0.27 ^a	1.59 ± 0.29 ^a
Diabetic + 0.3 µg/kg CB1093	99 ± 55 ^a	2.4 ± 0.3 ^a	51 ± 46 ^a	1.09 ± 0.65	1.75 ± 0.59 ^a
Diabetic + 1 µg/kg CB1093	162 ± 51 ^{a,b}	5.3 ± 1.6 ^b	134 ± 65 ^{a,b}	1.29 ± 0.19 ^{a,b}	1.95 ± 0.55 ^a

Data are means ± 1 SD ($n = 7$ for all groups); ^a denotes $p < 0.05$ vs equivalent (i.e. same drug treatment) controls, ^b denotes $p < 0.05$ vs vehicle-treated diabetic animals, ^c denotes $p < 0.05$ vs vehicle-treated control animals

so treated (Table 2). Treatment of non-diabetic rats with CB1093 reduced mechanical threshold ($p < 0.05$ vs vehicle-treated controls) to a level similar to that seen in diabetic rats treated with vehicle. Treatment of diabetic rats with CB1093 did not further reduce paw pressure thresholds (Table 2).

Nerve conduction velocity. Diabetic rats treated with vehicle showed the now “classical” reductions in both MNCV and SNCV compared to their control counterparts (Table 2). Neither dose of CB1093 affected these conduction velocity measurements in diabetic or control animals.

Steady-state NGF-LI concentrations in the sciatic nerve. Treatment with CB1093 produced similar ef-

fects on steady-state NGF-LI concentrations in sciatic nerve from control and diabetic animals. In control animals, CB1093 at a dose of 1 µg/kg was effective ($p < 0.05$) at increasing NGF-LI in the sciatic nerve (Table 3). Concentrations of NGF-LI were reduced ($p < 0.05$) in the sciatic nerve of untreated diabetic rats compared with untreated control rats (Table 3). Treatment with CB1093 (1 µg/kg), however, raised ($p < 0.05$) the NGF-LI in the sciatic nerve of diabetic rats to concentrations similar to those in untreated controls (Table 3). No statistically significant effect was seen with the lower dose in either group.

Steady-state neuropeptide concentrations in the sciatic nerve. In the control group, treatment with the higher dose of CB1093 increased ($p < 0.05$) the steady-state

Fig. 1. Northern blot showing expression of NGF mRNA in foot skin from 9 control (C) and nine diabetic (D) rats treated with vehicle (V) or 0.3 or 1.0 mg/kg CB1093. A fluorograph of the ethidium Br-stained gel is shown at the top

CGRP concentrations, with a non-significant increase in substance P (Table 3). Comparison of the control and diabetic animals that had received vehicle showed clear depletions in the content of substance P and CGRP in the sciatic nerve of untreated diabetic rats ($p < 0.05$). The higher dose of CB1093 (1 $\mu\text{g/kg}$) raised the sciatic nerve concentrations of substance P and CGRP ($p < 0.05$), so that they were similar to those of control rats treated with vehicle. No changes were observed at the lower dose of CB1093 in either group of rats.

Nerve growth factor-like immunoreactivity in the soleus and EDL muscles. The soleus muscle showed pronounced reductions in NGF-LI in diabetic animals treated with vehicle ($p < 0.05$ vs vehicle control; Table 3). In non-diabetic rats both doses of CB1093 raised the soleus NGF content ($p < 0.05$). In diabetic rats, CB1093 caused an increase ($p < 0.05$) in NGF-LI in the soleus muscle at a dose of 1 $\mu\text{g/kg}$. Although the effect of the low dose was not significant by comparison with either group treated with vehicle, both doses of CB1093 raised the mean NGF content of the soleus above that of control rats treated with vehicle. A reduction in NGF-LI was also seen in EDL muscle from diabetic rats (Table 3) but CB1093 treatment did not significantly alter the EDL muscle NGF-LI in control or diabetic animals.

Nerve growth factor mRNA hindlimb in the foot skin. Nerve growth factor protein content of foot skin from the hindlimb was below the limit of detection of the ELISA assay. Therefore, the foot skin was used for measurement of NGF mRNA by northern blotting (Fig. 1). There was considerable inter animal variation but clear reductions were always seen in skin from diabetic rats treated with vehicle and the high dose of CB1093 increased expression of NGF message, especially in diabetic rats (Fig. 1).

p75 and trkA protein concentrations in the dorsal root ganglia. The results from western blotting of p75 neu-

Fig. 2. Typical western blots showing immunoreaction products for p75^{NTR} and trkA on western blots from DRG homogenates from control and diabetic animals. Each lane represents an extract from the pooled L₄ and L₅ ganglia of a single rat

rotrophin receptor (p75^{NTR}) and trkA in the DRG are depicted in Table 3, with sample blots in Figure 2. The p75^{NTR} species in the DRG had an apparent weight of 75 and 65 kDa. The concentration of p75^{NTR} protein in the DRG of diabetic rats treated with vehicle was reduced by 41 % ($p < 0.05$) when compared with their control counterparts. Treatment with the high dose of CB1093 had no effect on p75^{NTR} in the DRG from control or diabetic animals (Table 3). Two major trkA species were present in the DRG with apparent weights of 145 and 110 kDa (Fig. 2). These were quantified to show expression of the primary translation product (110 kDa) and the fully glycosylated mature receptor (145 kDa). Comparison of the concentrations of the trkA species in the DRG from the control and diabetic rats that were treated with vehicle showed no significant change in any of the species and there was no effect of CB1093.

Discussion

Most of the findings reported here are explainable by the hypothesis that CB1093 stimulates the expression of NGF in both skin and muscle of control and diabetic rats. There were no effects on nerve conduction measures in fast-conducting large fibres because

Table 4. The effects of CB1093 on the NGF receptors, p75^{NTR} and trkA protein concentrations in dorsal root ganglia of control and diabetic rats

	p75 ^{NTR} protein	trkA protein	
	65 kDa + 75 kDa	110 kDa	145 kDa
Vehicle-treated Control	1.00 ± 0.16	1.0 ± 0.07	1.0 ± 0.2
Control + 0.3 µg/kg CB1093	ND	ND	ND
Control + 1 µg/kg CB1093	0.94 ± 0.17	1.0 ± 0.05	0.93 ± 0.1
Vehicle-treated Diabetic	0.56 ± 0.08 ^a	0.97 ± 0.05	0.93 ± 0.05
Diabetic + 0.3 µg/kg CB1093	ND	ND	ND
Diabetic + 1 µg/kg CB1093	0.59 ± 0.22 ^a	0.95 ± 0.14	0.93 ± 0.12

Data are means ± 1 SD ($n = 4$ for controls and $n = 5$ for diabetics). ^a denotes $p < 0.05$ vs equivalent (i.e. same drug treatment) controls. ND denotes not determined

these are not responsive to NGF [21]. There is no doubt that the genes coding for substance P and CGRP in primary afferent C fibres are responsive to NGF [22, 23] and that giving NGF exogenously to diabetic rats corrects sciatic nerve deficits in these two peptides [5]. Thus, it is safe to assume that correction of substance P and CGRP deficits by CB1093 is secondary to the increase in NGF production and its collection by the peripheral projections of sciatic sensory neurones. It is striking that the extent of the increase in sciatic nerve NGF closely paralleled that in soleus muscle but that the increases in neuropeptide concentrations were less pronounced. This could indicate that increased expression is maximised in controls at the levels attained with 1.0 mg/kg CB1093 and that, in diabetics, NGF signalling is sub-optimal or changed in diabetes. This latter possibility receives support from another study in which NGF treatment failed to influence p75^{NTR} immunoreactivity on western blots from the DRGs of diabetic rats [24]. It is also striking that NGF expression in the EDL muscle was refractory to the stimulatory effects of CB1093, though it was reduced in diabetes. The mechanisms by which CB1093 induces NGF are currently under investigation and we have no insight as yet into potential differences between muscle types. Although there is information on differential effects of diabetes on slow (soleus) and fast (EDL) muscle types, we have been unable to discover information on their neurotrophin production.

The mechanical hyperalgesia is puzzling but it is likely that different mechanisms operate in generating the phenomenon in CB1093-treated, non-diabetic rats and in diabetic rats treated with vehicle. A single injection of NGF (1 mg/kg, s.c.) has been reported to decrease paw pressure threshold in control animals [17]. Accordingly, the mechanical hyperalgesia in CB1093-treated controls could derive from increased endogenous NGF availability. This is, however, not an adequate explanation because it cannot be extended to the diabetic animals treated with vehicle, whose C fibres are NGF-deficient. The fibre population mediating mechanical nociception peripherally is mixed and not all components are NGF-dependent, so that

it is entirely feasible for a similar response to be produced by NGF-derived and by NGF-independent mechanisms, as has been suggested by others [25–29]. There is pharmacological evidence suggesting an involvement of *N*-methyl-D-aspartate glutamate receptors in the exaggerated response of diabetic rats and this mechanism is not NGF-mediated [30]. Thus, we suggest that the two phenomena differ in origin and that their not being additive in CB1093-treated diabetic rats does not preclude different mechanisms.

It is also pertinent to consider hypercalcaemia as a potential cause of neurophysiological defects in association with CB1093 treatment. Studies in one laboratory (Miller, personal communication) show, however, no statistically significant increase in plasma Ca^{2+} with oral treatment at $3 \mu\text{g} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$ [108.6 ± 1.2 (SEM)% of controls]; this was three times the dose used here. Hence, it is unlikely that hypercalcaemia caused hyperexcitability of nociceptors.

The observations reported here prompt the question of a potential physiological role for vitamin D in the regulation of NGF-derived neurotrophic support. This is especially pertinent for skeletal muscle; because it is a major source of NGF, because there is evidence for a physiological role for vitamin D in other aspects of muscle physiology and because of the cross-talk between neuropathies and myopathies in diabetes mellitus. Degradation of skeletal muscle in vitamin D deficiency is an old observation [31] and 1,25-(OH)₂D₃ is known to influence Ca^{2+} handling and related metabolic events in a fashion that indicates a physiological role [32–34]. It is also interesting that analogues of 1,25-(OH)₂D₃ have been shown to stimulate Ca^{2+} uptake and DNA synthesis in myocytes, such that structural modification of these molecules appears to increase their therapeutic potential for the treatment of myopathies [35]. Altered vitamin D metabolism has been shown in both short-term and long-term diabetes [36, 37] and decreased concentrations of 1,25-(OH)₂D₃ are commonly observed, despite the different calcium balances. It is, therefore, possible that regulation of neurotrophic support through the expression of NGF form part of the phys-

iological role of vitamin D and that disturbances of its metabolism in diabetes contribute to the development of neurotrophic deficits as a prelude to diabetic neuropathies. Thus, reversal of neurotrophic deficits by analogues of vitamin D could represent corrective therapy rather than simply a pragmatic solution to the problem. In any event, the efficacy of this potent orally active compound argues well for a pharmacological approach to neurotrophic deficits in diabetes and in other disease states.

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