High concentrations of dichloroacetate have minor effects on the vitality of the mammalian nerve fibers: an ex-vivo electrophysiological study

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Dichloroacetate has been used extensively in the treatment of cancer and genetic mitochondrial diseases, but there have been reports of dichloroacetate-induced peripheral neuropathy. In this study, the acute effects of sodium dichloroacetate on the peripheral nerve fibers were investigated, using an ex-vivo preparation, in the isolated sciatic nerve of the rat. The amplitude of the evoked nerve compound action potential (CAP) was measured to confirm the proper functioning of the nerve fibers. The half-vitality time [the time required to decrease the CAP to 50% of its initial value, here called inhibitory time 50% (IT_{50})], of the nerve fibers, which had been incubated in normal saline, was 30.4 ± 0.26 h ($n = 12$). When the nerve fibers were incubated in 10 mmol/l of dichloroacetate, the IT_{50} was 29.7 ± 0.34 h ($n = 8$), with no significant difference from the control ($P > 0.05$). The fact that such a high concentration of dichloroacetate as 10 mmol/l had no effect on the parameters of the evoked CAP is an indication of the high tolerance of peripheral nerve fibers to this compound. When a concentration of 20 mmol/l of dichloroacetate was tested, a 15.2 ± 1.25% ($n = 12$) inhibition in the CAP amplitude occurred, but although a relatively small population of nerve fibers was inactive, the vitality of the remaining active axons was not affected, with a final IT_{50} of 28.1 ± 0.64 h ($n = 12$), with no significant difference from the IT_{50} of the control, which for this group of experiments was 28.1 ± 0.17 h ($P > 0.05$). This moderate effect, with a 15.2 ± 1.25% decrease in the CAP amplitude, suggests that within the exposure limitation of the sciatic nerve preparation of 28–30 h, there could be a gradual development of certain biochemical changes leading to the early stages of dichloroacetate-induced neurotoxicity. *Anti-Cancer Drugs* 22:273–276 © 2011 Wolters Kluwer Health | Lippincott Williams & Wilkins.

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Introduction

Dichloroacetate has been used for several years as an investigational drug in the treatment of different forms of cancer [1] and in a variety of acquired and congenital metabolic disorders [2,3]. However, chronic administration of dichloroacetate, over a period of weeks to months, has been blamed for peripheral neuropathy in humans and laboratory animals at or above clinically relevant doses [4–8]. In-vitro studies conducted on isolated primary cultures of Schwann cells and dorsal root ganglia neurons of rat have shown that exposure to 1–20 mmol/l of dichloroacetate for 12 days decreased the expression of several major peripheral nervous system myelin proteins and this effect was only partially reversible after dichloroacetate washout [9]. The lack of a nerve tissue model that approximates dichloroacetate-induced neurotoxicity prompted us to carry out a detailed characterization of the function of the peripheral nerves in the presence of dichloroacetate. The purpose of this study was to assess and quantify the acute effects of high concentrations of dichloroacetate, of 10 and 20 mmol/l respectively, on the peripheral nervous system of mammals, using the isolated rat sciatic nerve.

Methods

The sciatic nerves of male rats (250–300 g) were dissected, from the spinal cord to the knee, and the epineurium and the perineurium were removed to maximize access of the drug to all nerve fibers. The nerve was mounted across a three-chambered recording bath, made of Plexiglas, identical to the one used in a number of neurotoxicological studies [10,11]. Each chamber was filled with 12 ml of the saline solution that was oxygenated (O_2, 100%) once, at the beginning of the experiment, and was gently stirred throughout the experiment (for over 30 : 00 h). The nerves were immersed in a modified Krebs–Ringer saline solution, containing (in mmol/l) NaCl (136), KCl (4.7), $CaCl_2$ (2.4), $MgCl_2$ (1.1), $NaHCO_3$ (1), glucose (11.0), and 4-(2-hydroxyethyl)-1-piperazine ethane sulfonic acid (10; pH = 7.2; temperature 26.0 ± 1°C). When required, 11 mmol/l of glucose were replaced with 5 mmol/l. All experimental procedures were performed in accordance with the protocols outlined by Aristotle University of Thessaloniki, Greece and the local Veterinary Authorities (license no for the specific experiment 13/4712-29.04.2010), with regard to the recommended standard practices for biological investigations.

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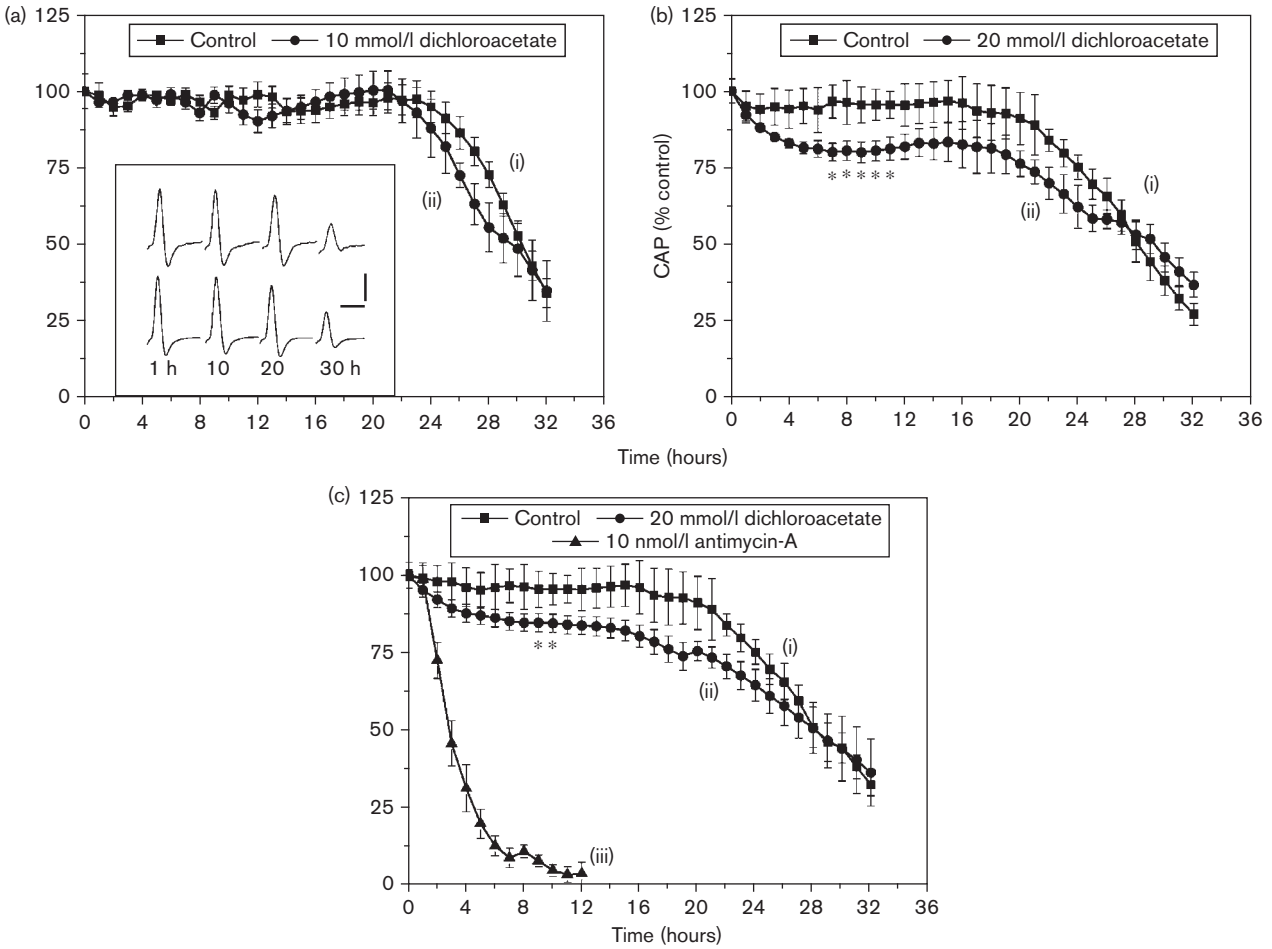
To monitor the vitality of the sciatic nerve fibers in the three-chambered recording bath, their evoked compound action potential (CAP) was recorded throughout the experiment (Fig. 1a insert records, upper trace), using standard electrophysiological stimulating and recording methods. The amplified analog CAP was digitized (25.0 kHz) using a data acquisition interface (Keithley KPCI-3102; Keithley Instruments, Cleveland, Ohio, USA) and analyzed using the appropriate software (Labview 5.1, National Instruments, USA). In the case of supramaximal stimulation, the CAP reflects the sum of the action potentials of all the synchronously stimulated sciatic nerve fibers. From the whole waveform of the CAP, it is clear that only 5–7% belongs to the electrical activity of C-fibers, calculated using the method described elsewhere [12], and the rest is activity from the A-fibers. Thus, the CAP amplitude measured in volts, from baseline to peak, can be used as an index of neuronal viability. In this case, the CAP amplitude was used to quantify the vitality of the sciatic nerve fibers exposed to normal saline or dichloroacetate.

Results

The main advantage of the three-chambered bath is that when the nerve is incubated in normal saline, the waveform of the evoked maximum CAP remains constant

Fig. 1



(a) The insert records represent recordings of the evoked compound action potential (CAP) from the isolated sciatic nerve of the rat in the three-chambered recording bath. Upper records the CAPs of the sciatic nerve exposed to normal saline. Lower records the CAP of the sciatic nerve fibers exposed to 10 mmol/l of dichloroacetate. The CAPs are sample records obtained every 10 h. Vertical scale bar: 2 mV; horizontal scale bar: 1.5 ms. From sample records of the CAP, such as those shown in the insert diagram, but obtained every hour, the vitality curves were produced for nerves incubated in normal saline [curve (i)] and those exposed to 10 mmol/l of dichloroacetate [curve (ii)]. The vitality curves represent the percentage decrease in the amplitude of the evoked CAP of the isolated sciatic nerve fibers as a function of the incubation time. In the case of dichloroacetate incubation, 100% constitute the amplitude (mV) of CAP before the application of the compound. Each data point represents group of the percentage values measured every hour and expressed as a mean ± standard error of the mean to plot the vitality/the time–response curves ($n=12$). (b) As in (a), the curves show the changes of the evoked CAP, in the presence of normal saline [curve (i), $n=12$] and in 20 mmol/l of dichloroacetate [curve (ii), $n=12$]. *Significant differences ($P<0.05$). (c) Curve (i), the nerve was incubated in saline containing 5 mmol/l of glucose ($n=11$). Curve (ii), the nerve was incubated in 20 mmol/l of dichloroacetate diluted in saline containing 5 mmol/l of glucose ($n=11$). Curve (iii), the sciatic nerve was incubated in normal saline (11 mmol/l glucose) with antimycin-A. The final concentration of antimycin-A was 10 nmol/l ($n=7$). *Significant differences ($P<0.05$).

for over 25:00 h (Fig. 1a, insert records, upper trace, times $t = 1$, 10, and 20 h). After that, there is a gradual decrease in the CAP amplitude because of natural nerve fiber inactivation (see record at time $t = 30:00$ h). The vitality of the nerve fibers was shown more accurately using the time–response curve, which represents the CAP amplitude versus time curve [see Fig. 1a, curve (i)]. To obtain this curve, the values of the CAP amplitude were measured (in volts) at a specific time (every hour), and the values of different experiments were averaged and finally expressed as mean ± standard error of the mean. These values were then expressed as the percentage of the CAP, at time $t = 0$ for the control, or the time before the application of dichloroacetate, which in both cases was considered to be 100%. Using the percentage values for each hour, it was possible to plot the time–response curve (vitality curve) for nerves exposed to normal saline [see Fig. 1a and b, curve (i)]. From these time–response curves for the control nerves and those exposed to dichloroacetate, it was possible to estimate the time required to decrease the CAP to 50% of its initial value (at time $t = 0$), here called inhibitory time 50% (IT_{50}), using the program GraphPad Prism 5.0 (GraphPad Software, Inc., San Diego, California, USA).

When the sciatic nerve was incubated in saline in which 10 mmol/l of dichloroacetate had been diluted (1–20 mmol/l were used *in vitro* by Felitsyn *et al.*, 2007), there was no effect on the CAP amplitude during the whole exposure period (Fig. 1a insert diagram, lower records) compared with the control (upper records). As was expected, the time–response curves for the nerves exposed to 10 mmol/l of dichloroacetate [Fig. 1a curve (ii)] and those exposed to normal saline [curve (i)] were identical. Thus, the IT_{50} for the nerves exposed to 10 mmol/l DCA was estimated to be 29.7 ± 0.34 h ($n = 12$), whereas the IT_{50} for the control nerves was 30.4 ± 0.26 h ($n = 12$). A comparison of the IT_{50} values, using the unpaired *t*-test, showed that there was no significant difference ($P > 0.05$; GraphPad Instat). This is an indication that the concentration of 10 mmol/l of dichloroacetate has no effect on the CAP amplitude or on the vitality of the sciatic nerve fibers, even after continuous exposure for 30:00 h.

When the nerve was exposed to a much higher concentration of dichloroacetate, of 20 mmol/l, there was a gradual decrease in the CAP amplitude to 15.2 ± 1.25% ($n = 12$) of the control values [Fig. 1b compare curves (i) and (ii)]. This decrease in amplitude was significant compared with the control curve (i) ($P < 0.05$, unpaired *t*-test), as marked by the asterisks on curve (ii). This is an indication that a relatively small population of the sciatic nerve fibers was inactivated and that the vitality of the remaining active nerve fibers was not affected by such a high concentration of dichloroacetate. The IT_{50} of the remaining nerve fibers was 28.1 ± 0.64 h [$n = 12$; derived from curve (ii) in Fig. 1b], which is not significantly different when compared with the control ($P > 0.05$, unpaired *t*-test). In this group of experiments the IT_{50} for the control was estimated to be 28.1 ± 0.17 h, as derived from curve (i) in Fig. 1b.

Furthermore, apart from the mild inhibitory effects on the amplitude of the CAP at 20 mmol/l, neither 10 nor 20 mmol/l of dichloroacetate had any effect on the duration of the CAP, a parameter related to the functioning of the voltage-gated sodium channels and voltage-gated potassium channels of the nerve fibers. The duration of the CAP was defined as the time interval between the onset of the positive peak and return to the baseline (Fig. 1a insert records). In nerves exposed to normal saline, the duration of the CAP was 1.11 ± 0.03 ms at time $t = 1:00$ h and 1.24 ± 0.08 at time $t = 20:00$ h ($n = 12$). There was no significant difference between the values of duration measured at time $t = 0$ and $t = 20:00$ h ($P > 0.05$, unpaired *t*-test, $n = 12$). The situation was similar regarding the nerves incubated in either 10 or 20 mmol/l of dichloroacetate.

Discussion

In this study, the sciatic nerve was incubated in a modified Krebs–Ringer saline solution, containing 11 mmol/l of glucose. This is a relatively high concentration of glucose, near or above the levels of hyperglycemia, as normal glucose levels in the plasma of mammals is between 2.5 and 5 mmol/l. Dichloroacetate is effective in reducing hyperglycemia in chemical or surgically induced experimental diabetes [2]; thus, it is possible that a more significant effect of dichloroacetate on the functioning of the sciatic nerve is being offset by the high glucose levels. Therefore, equivalent data using a modified Krebs–Ringer saline solution with 5 mmol/l of glucose, were provided for further clarification. The effects of 20 mmol/l of dichloroacetate on the CAP amplitude of a group of nerves ($n = 11$) incubated in saline with 5 mmol/l of glucose [Fig. 1c curve (ii)], were found to be almost identical to those obtained from the same concentration of dichloroacetate diluted in saline containing 11 mmol/l [see Fig. 1b curve (ii)]. In both cases, for the first 24:00 h of incubation in dichloroacetate, there was a gradual decrease in the CAP amplitude to 15.2 ± 1.25% for the saline with 11 mmol/l [Fig. 1b curve (ii)] and 14.6 ± 1.23% [Fig. 1c curve (ii); $n = 11$] for saline with 5 mmol/l of glucose. Furthermore, the IT_{50} values were almost identical in both glucose concentrations (28.2 ± 0.10 h, $n = 11$). The decrease in the CAP in both cases, was significantly different compared with the control (saline without dichloroacetate; see asterisks in Fig. 1b and c, $P < 0.01$). This is an indication that the slight toxic effect observed in both cases, of 5 and 10 mmol/l of glucose, is not related to the levels of glucose in the saline.

The question here is whether the preparation of the isolated rat sciatic nerve is sensitive enough for such neurotoxicological studies. For comparison, as a negative control the compound antimycin-A, which is a complex

III inhibitor [13], was used. Antimycin-A seems to be extremely neurotoxic, as 10 nmol/l eliminated the CAP completely within 12 : 00 h [Fig. 1c, curve (iii)], with an IT_{50} of 2.6 ± 0.03 h ($n = 6$), calculated from curve (iii).

The two concentrations of dichloroacetate used in this study, of 10 and 20 mmol/l, respectively, are extremely high compared with antimycin-A. They are high even when compared with those concentrations used in clinical treatment, as the concentration of 1 mmol/l is similar to the peak plasma levels achieved in patients who receive 50 mg/kg doses of the dichloroacetate intravenously. Furthermore, in experiments with a 1-day oral application of 50 mg/kg, the plasma concentration was found to have a peak of 0.330–0.594 mmol/l [14]. In addition, it is worth noticing that in both cases, dichloroacetate was eliminated from the plasma within 12 h [14]. These in-vivo experiments, in human or animals, are more reliable compared with ex-vivo studies, limited to 30 : 00 h incubation, because of a much longer exposure period to dichloroacetate. *In vivo*, the oral dichloroacetate treatment (50–500 mg/kg per day) can last a few weeks (16 weeks) [8] to a few months [5,6]. However, even in such long-term treatments, for example, of 4 weeks of dichloroacetate (500 mg/kg), electrophysiological testing and morphometric analysis indicated a slowing of the large myelinated motor and sensory fiber nerve conduction velocity, but not axonal death [8].

In our experiments, lasting usually up to 30 h, to compensate for the difference between short-term and long-term experiments, high concentrations of dichloroacetate, of 10 and 20 mmol/l, were used to accelerate possible toxic effects. Even at these high concentrations, the effects on the vitality of the nerve fibers were minimal, compared with other compounds, with a specific target of action, tested on the same nerve preparation. For example, antimycin-A, at a concentration of approximately 10^6 times lower than the concentrations of dichloroacetate used in this study, eliminated the CAP within 12 : 00 h [Fig. 1c curve (iii)]. At concentrations of 100 µmol/l, oxaliplatin, an anticancer drug that causes severe peripheral neuropathy, was found to cause a 2310% increase in the repolarizing phase of the CAP within 3–6 h, obviously affecting the voltage-gated potassium channels [11]. Finally, cadmium, at a concentration of 250 µmol/l, tested in an identical recording chamber, was found to eliminate the CAP within 10–12 h. This was a neurotoxic effect caused mainly by the increase in reactive oxygen species [15], an effect that was reversed by 1.0 mmol/l of *N*-acetyl-L-cysteine [16]. None of these acute effects occurred during the exposure of the rat sciatic nerve fibers to 10 mmol/l of dichloroacetate, whereas only a 15.2 ± 1.25% decrease in the amplitude of the CAP occurred at 20 mmol/l, without affecting the vitality of the remaining sciatic nerve fibers. This is a clear indication that dichloroacetate, within the recording and exposure limitation of the sciatic nerve preparation, has minor effects on the vitality of the sciatic nerve fibers, assessed using their compound action potential. However, the moderate effect of 20 mmol/l of dichloroacetate, with a 15.2 ± 1.25% decrease in the CAP amplitude, suggests that within the exposure limitation of the sciatic nerve preparation, there could be a gradual development of biochemical changes leading to the early stage of dichloroacetate-induced neurotoxicity. For this reason we believe that this particular nerve preparation can be further improved by combining the electrophysiological data presented here with an extensive biochemical study.

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