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Comparison of Carbon Dioxide, Argon, and Nitrogen for Inducing Unconsciousness or Euthanasia of Rats

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We compared CO_2, Ar, and N_2 for inducing unconsciousness and euthanasia of Sprague-Dawley rats. We determined time to unconsciousness and monitored heart rate (HR) and mean arterial blood pressure (MAP) by radiotelemetry to assess stress, recovery after exposure, and time of death. Unconsciousness (mean ± standard error) occurred 24 ± 3, 87 ± 8, and 93 ± 8 s after short-term exposure to CO_2, Ar, and N_2, respectively. During exposure, CO_2 depressed HR, whereas Ar and N_2 increased HR. Upon removal from the chamber, rats' HR rapidly normalized after CO_2 or N_2 but remained elevated for 60 min after Ar. During exposure, all agents depressed MAP, which returned to resting levels 10 to 50 min after rats' removal from the chamber. For euthanasia, CO_2 at approximately 100% induced unconsciousness in 37 ± 3 s, increased and then depressed MAP and HR, and caused death at 188 ± 15 s. CO_2 at approximately 30% induced unconsciousness in 150 ± 15 s, decreased HR and MAP, and induced death at 440 ± 9 s. Ar at approximately 100% increased MAP but decreased HR, induced unconsciousness with hyperreflexia at 54 ± 4 s, and caused death at 197 ± 20 s. N_2 at approximately 100% decreased MAP but not HR and produced unconsciousness with hyperreflexia at 164 ± 17 s and death at 426 ± 28 s. We conclude that CO_2 effectively produced unconsciousness and euthanasia, but we were unable to ascertain distress. Ar also appears effective but produced hyperreflexia and tachycardia. N_2 was ineffective.

Abbreviations: HR, heart rate; MAP, mean arterial blood pressure

High concentrations of CO_2 are commonly used to euthanize and produce unconsciousness in rodents, and comprehensive studies have been published on the use of this gas for these purposes.[3,5,7,9,12,13] However, some have proposed that Ar may be superior to CO_2 for this use.[11] Unlike CO_2, Ar does not irritate mucous membranes, and animals do not show aversive behaviors when first exposed to Ar as they do when initially exposed to CO_2.[11] There is some controversy in the literature regarding aversion upon exposure, because there are reports that CO_2 does not produce distress in rats.[2,3,5] As a result, we undertook the current study to compare CO_2, Ar, and N_2 for inducing unconsciousness and euthanasia of rats by using cardiovascular responses, determined with radiotelemetry, to determine the time course of action of the gases, follow recovery or progression to death, and monitor distress.

Materials and Methods

Animals. Adult male outbred rats (Holtzman Sprague-Dawley) were purchased from Harlan Sprague-Dawley (colony #205, Indianapolis, IN) at 200 to 224 g body weight. Rats used for all groups were reported by the vendor to be free from adventitious viruses, *Mycoplasma pulmonis*, respiratory and enteric bacteria, and ecto- and endoparasites at the time of purchase.

The rats were allowed to acclimate to the animal room conditions and husbandry procedures for 2 wk prior to surgical implantation of the radiotelemetry transmitters. The environmental conditions in the animal room were as follows: lighting, 200 lux at cage level (lights on, 0700 to 1900); temperature, 22 to 26 °C; and relative humidity, 30% to 60%. Animals were housed individually in conventional solid-bottom polycarbonate cages (Lab Products, Seaford, DE; nominal floor area with bedding, 930 cm^2) with standard stainless steel lids and hardwood chip bedding (depth, 4 to 5 cm; Sanichip, PJ Murphy Forest Products, Montville, NJ). Rat chow (Purina #5001, Purina Mills, Richmond, IN) and tap water were provided ad libitum.

Surgical procedures. A radiotelemetry transmitter (model TA11PA-C40, Data Sciences International, St Paul, MN) was aseptically implanted in the abdominal cavity via a 5- to 6-cm ventral midline incision of each rat under ketamine (80 mg/kg intraperitoneally; Ketaset, Ft Dodge Animal Health, Ft Dodge, IA) and xylazine (7 mg/kg intraperitoneally; Rompun, Bayer, Shawnee Mission, KS) anesthesia. The catheter attached to the telemetry transmitter was tunneled through the abdominal wall to a point near the skin incision in the left femoral triangle and inserted centrally into the femoral artery to a depth of 3.0 cm.

All animals were given 10 cc of sterile 5% dextrose subcutaneously immediately after surgery to prevent dehydration and to provide a short-term glucose supplement. Ketoprofen (Ketofen, Ft Dodge Animal Health, Ft Dodge, IA) was added to the 5% dextrose solution and administered at 16 mg/kg as described previously.[8]

Postsurgical recovery was monitored by daily visual examination of the incisions and overall condition of the rats and calculation of daily food and water intake; body weight was determined every other day; and mean arterial blood pressure (MAP), heart rate (HR), and movement in the cage were measured every 5 min by telemetry. All procedures were approved by the Wayne State University Institutional Animal Care and Use Committee.

Experimental procedures. Two studies were conducted us-

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ing CO_2, Ar, and N_2 obtained as nominal 100% concentrations (Cryogenic Gases, Detroit, MI).

In the 1st study, 9 rats were exposed individually to each of the 3 gases (on separate days with 2 to 3 days of rest in between exposures) in a large (66 L) acrylic chamber until they appeared unconscious (no head or limb movements), at which point they were removed and allowed to recover in their home cages. CO_2, Ar, and N_2 were introduced into the chamber by fully opening the value from the gas supply tank for 1 to 2 min prior to placing the rat into the chamber. No gas was introduced during the time the rat was in the chamber. Flow rates and concentrations of these gases were not determined, but the exposure procedure was the method that is recommended and used routinely at this institution. On a separate occasion, the same 9 animals were placed individually in the chamber containing room air for 1 min to determine the effects of exposure to the chamber itself.

In the 2nd study (euthanasia study), the rats in 3 groups (n = 5 to 7) were exposed individually to CO_2, Ar, or N_2 in the 66-L chamber until 1 min after respiration ceased. For these 3 exposures, gas was introduced at 10 L/min for 30 min prior to placing the 1st rat in the chamber to achieve a concentration of >99%, and flow was continuous while the rat was in the chamber and between rats. An additional group of 7 rats was euthanized with CO_2 in the 66-L chamber precharged for 2.5 min at 10 L/min to a calculated initial CO_2 concentration of 30%. Flow was not continued after the animal was placed in the chamber, and the chamber was flushed with compressed air for 2 min between rats. Direct measurement of gas concentrations was not done due to the lack of the appropriate analytical equipment. The exposure period to achieve desired gas concentrations was calculated using the equation Exposure time = chamber volume (L)/gas flow rate (L/min) × –ln(100 – desired concentration)/100, which is used by inhalation toxicologists to calculate gas concentrations in chambers of known volume.[4] Another 9 rats were placed individually for 8 min in the chamber filled with room air, for use as controls.

Collection of radiotelemetry data. Output from the telemetry transmitters was collected using hardware and software from Data Sciences International Corporation (St Paul, MN). Prior to rats' exposure to the gases, data were sampled for 10 s every 5 min. During and after the rats' exposure to gases, data (HR, MAP, movement) were collected every 20 s in the 1st study or every 6 or 10 s in the 2nd study. These data were saved to the hard drive of a desktop computer and subsequently transferred as Excel (Microsoft, Redmond, WA) spreadsheet files to other computers for summarization and statistical analyses.

Statistical analysis. The data are reported as mean ± standard error of the mean (SEM). The differences in the data across the postexposure period of the 1st study were analyzed using a general linear model analysis of variance with repeated measures and post-hoc contrasts (SPSS, Chicago, IL). Data collected within each treatment during the exposure period in the 1st study and during the 1st min of exposure in the 2nd study were analyzed by 1-way analysis of variance followed by the Dunnett post-hoc test using SigmaStat statistical software (Systat, Point Richmond, CA). Means were declared statistically significant at $P \leq 0.05$.

Results

Short-term exposure to unconsciousness. HR and MAP responses of male rats briefly exposed to CO_2, Ar, or N_2 are shown in Figures 1 and 2. For CO_2, the time required for rats to become unconscious, as judged by visual inspection, was 24 ± 3 s. During the exposure period, HR was significantly ($P < 0.05$) depressed compared with the control period but then returned to control levels by 80 s after the rats were removed from the exposure chamber and placed in their home cages. MAP also was significantly ($P < 0.05$) decreased below resting levels while rats were exposed to CO_2, but upon their removal from the exposure chamber, MAP was significantly ($P < 0.05$) increased for about 20 min.

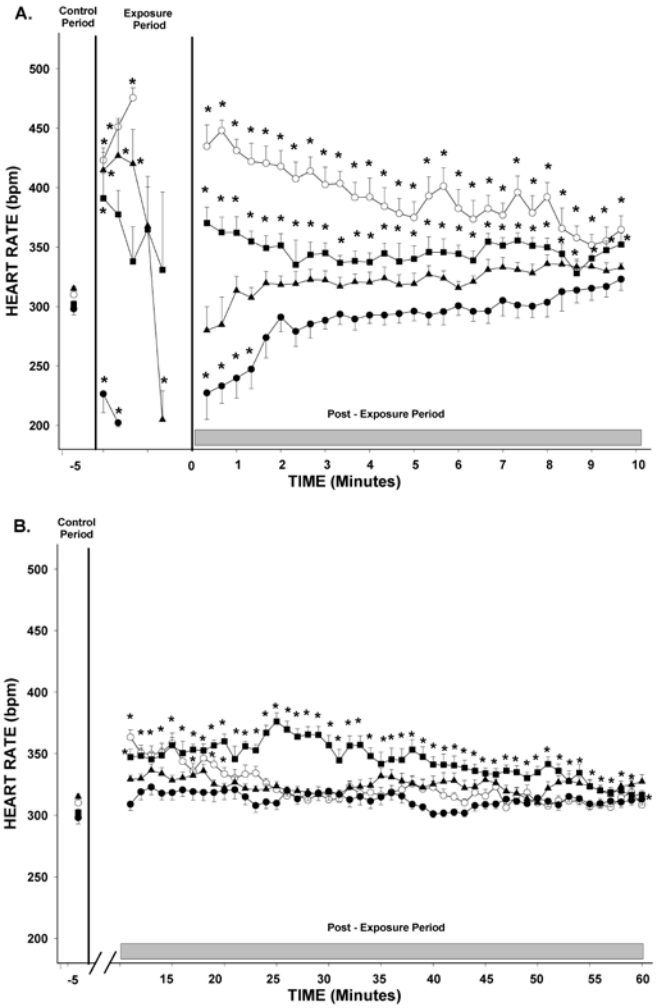

Figure 1. Effect of short-term exposure to air (○), CO_2 (●), Ar (■), and N_2 (▲) on heart rate (mean ± SEM; n = 7 to 9) of male Sprague-Dawley rats (A) before, during, and for 10 min immediately after exposure or (B) 10 to 60 min after exposure. 'Control period' is the 5-min period immediately prior to removal of each rat from its home cage for placement in the exposure chamber. 'Exposure period' is the period required to achieve apparent unconsciousness (gases) or 1 min (air only); the exposure period is not shown in (B). 'Postexposure period' is the 60-min period immediately after removal of rats from the exposure chamber. *, Significantly different ($P < 0.05$) versus values during control period.

Acute exposure to Ar caused unconsciousness in 87 ± 8 s. HR was significantly ($P < 0.05$) elevated during exposure and remained so for 60 min after the animals were returned to their home cages. MAP also was significantly ($P < 0.05$) increased during exposure and remained so for about 20 min after the animals were removed from the chamber.

N_2 produced unconsciousness in 93 ± 8 s. During exposure, HR was significantly ($P < 0.05$) increased then decreased, whereas MAP was significantly ($P < 0.05$) decreased. Upon rats' removal from the gassing chamber, HR returned to control levels within 20 s, but MAP remained significantly ($P < 0.05$)

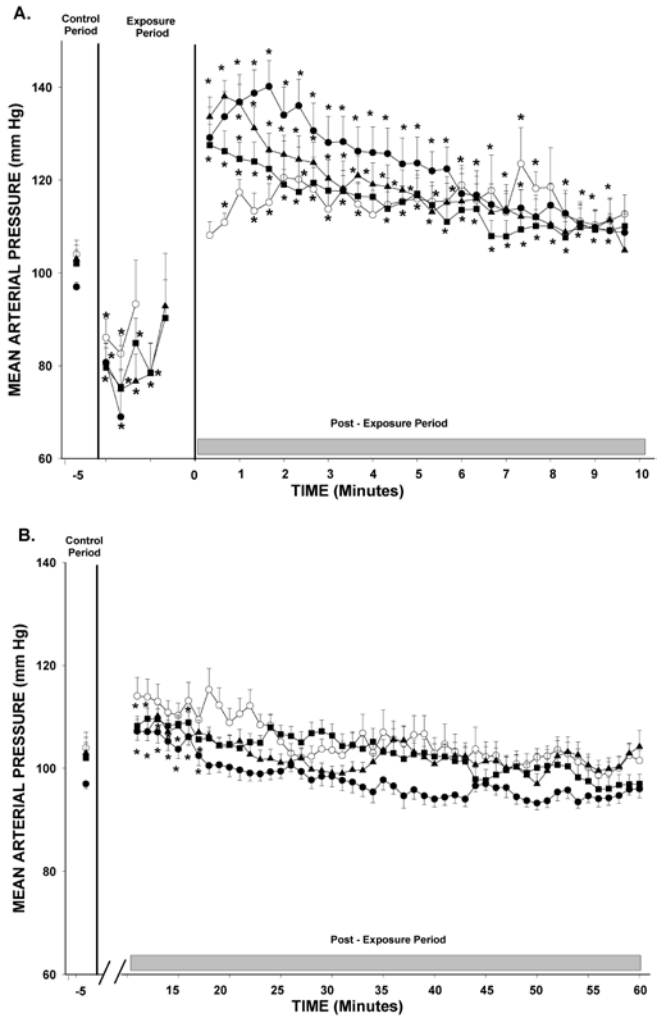

Figure 2. Effect of short-term exposure to air (○), CO_2 (●), Ar (■), and N_2 (▲) on mean arterial blood pressure (MAP; mean ± SEM; n = 7 to 9) of male Sprague-Dawley rats (A) before, during, and for 10 min immediately after exposure or (B) 10 to 60 min after exposure. 'Control period' is the 5-min period immediately prior to removal of rat from its home cage for placement in the exposure chamber. 'Exposure period' is the period required to achieve apparent unconsciousness (gases) or 1 min (air only); the exposure period is not shown in (B). 'Postexposure period' is the 60-min period immediately after removal of rats from the exposure chamber. *, Significantly different ($P < 0.05$) versus values during control period.

elevated for 5 min.

Exposure to the chamber filled with room air for 1 min significantly increased HR but unexpectedly reduced MAP. After the rats were returned to their home cage, HR and MAP remained significantly ($P < 0.05$) elevated for about 20 and 8 min respectively.

Rats acutely exposed to CO_2 were relaxed and nonresponsive to touch and handling. In contrast, rats exposed to Ar and N_2 exhibited muscle spasms and were hyperreflexic to touch and handling when they appeared unconscious.

Euthanasia study. Figure 3A through C shows HR, MAP, and movement in the chamber when rats were exposed continuously to calculated 100% concentrations of CO_2, Ar, or N_2 until animals were euthanized. Figure 3D shows the cardiovascular and activity responses of rats exposed to the chamber initially charged to approximately 30% CO_2. Figure 4 shows the HR and MAP data collected during the 1st minute of these exposure periods. Exposure to an air-filled chamber for 8 min was done, but the data are not shown.

CO_2 at a calculated concentration of 100% increased MAP and movement in the chamber during the first 12 s of exposure, but then MAP declined and was significantly ($P < 0.05$) depressed from 24 to 60 s of exposure (Figure 4B). HRs of rats exposed to 100% CO_2 were not increased initially. Instead, HR declined significantly ($P < 0.05$) at approximately 40 s of exposure (Figures 3A and 4A). Apparent unconsciousness was achieved at 37 ± 3 s, and death occurred at 188 ± 15 s (Figure 3A). Exposure to approximately 30% CO_2 (Figures 3D and 4) significantly ($P < 0.05$) depressed HR within 10 s. MAP was slightly, but not significantly, increased at 10 s and remained unaltered until about 6 min of exposure. Apparent unconsciousness occurred at 150 ± 15 s, and death occurred at 440 ± 9 s.

Exposure to Ar produced no significant change in HR until 55 s, at which time HR significantly ($P < 0.05$) decreased. MAP was significantly ($P < 0.05$) increased for the 1st minute of Ar exposure. Movement in the cage was more prolonged than was observed for CO_2; apparent unconsciousness was achieved in 54 ± 4 s, and death occurred at 197 ± 20 s (Figure 3B and 4).

N_2 exposure produced no significant changes in MAP during the 1st minute, whereas HR was significantly ($P < 0.05$) depressed after about 55 s. The increase in movement in the chamber was similar to that observed with Ar. Apparent unconsciousness occurred at 164 ± 17 s, and death occurred at 426 ± 28 s after N_2 exposure (Figures 3C and 4).

Exposure to room air in the chamber induced sustained and significant ($P < 0.05$) increases in HR, MAP, and movement for the entire 8-min exposure period (data not shown).

Discussion

The current data show that a high concentration of CO_2 (calculated to be approximately 100%) acts rapidly and effectively to induce unconsciousness and euthanasia of rats—findings that are not new. Precharging the gassing chamber with CO_2 to a lower initial concentration (approximately 30%) as suggested in the AVMA Panel on Euthanasia[1] also induced unconsciousness and death without obvious signs of distress to the rats, but the times required to induce both states were much longer. Ar at approximately 100% acted almost as rapidly as CO_2 but produced hyperreflexia and prolonged tachycardia following short-term exposure. N_2 at approximately 100% was not very effective, as it was slow to produce unconsciousness and death and induced hyperreflexia during short-term exposure.

Although the exact gas concentrations in the chamber were not determined in the current studies, MAP and HR data collected during inhalation of the higher calculated concentration of CO_2 are in good agreement with the report of Smith and Harrap,[12] who exposed rats to a chamber prefilled with CO_2 to a measured concentration of 75%. In addition, times to unconsciousness and cardiovascular responses in the 1st study (Figures 1 and 2), in which flow rate was high but not controlled, were in general agreement with those in the 2nd study (the euthanasia study; Figures 3 and 4), in which flow rate was controlled at 10 L/min and concentrations of gas were calculated.

Concerning the question of whether CO_2 causes stress, neither the previous report[12] nor the present data provide the answer. The initial increase in MAP in CO_2-exposed rats in the current study may be a sign of stress, especially when linked to the aversive behavior reported by others.[11] In addition, it has been previously shown that hypoxic hypercapnia, which would be expected in animals breathing approximately 100% CO_2,

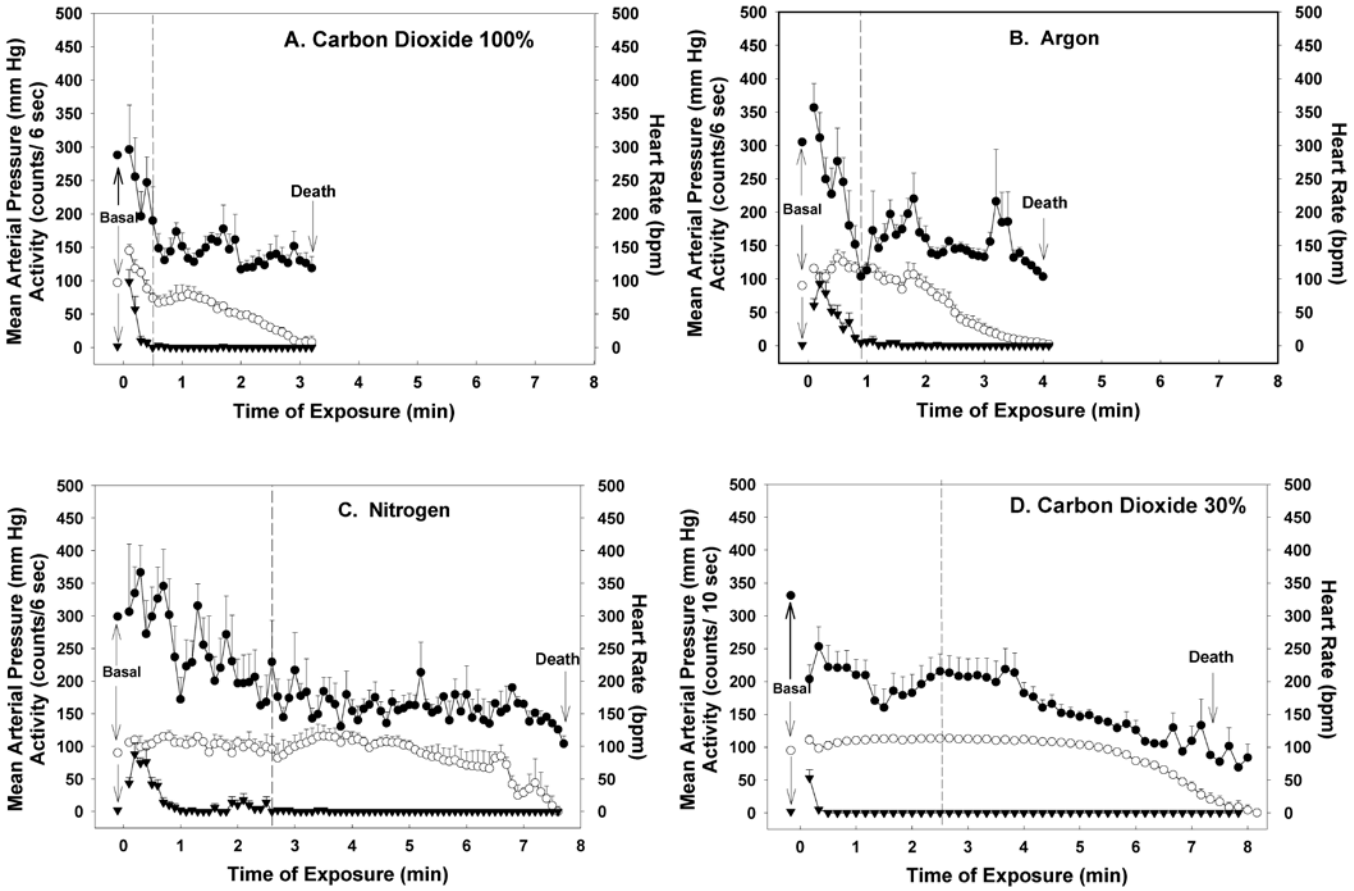

Figure 3. Effect of exposure to (A) 100% CO_2, (B) Ar, (C) N_2, or (D) 30% CO_2 on heart rate (●), mean arterial blood pressure (○), and activity (▲; mean ± SEM; n = 5 to 9) of male Sprague-Dawley rats in the euthanasia study. The concentrations of CO_2 shown (30% or 100%) are the initial calculated concentrations to which the chamber was precharged. For Ar and N_2, the initial concentrations were calculated to be 100%. The dotted line in each panel indicates the mean time at which apparent unconsciousness occurred.

increased blood pressure and peripheral sympathetic neural activity.[6,10] However, we observed that HR was depressed by CO_2 exposure, and this finding together with the changes in MAP are consistent with activation of peripheral and central chemoreflexes. In this regard, the present data are in excellent agreement with those of Hirakawa and colleagues,[10] who showed that moderate hypoxic hypercapnia produced by exposing rats to an environment of 10% O_2 and approximately 7% CO_2 increased blood pressure and depressed heart rate—responses that could be blocked by sinoatrial denervation, which removes the afferent limbs of peripheral baro- and chemoreflexes. Therefore, using HR and MAP to detect stress during exposure to high concentrations of CO_2 is severely limited by the confounding responses induced by activation of chemoreflexes.

That exposure to approximately 30% CO_2 rapidly and significantly depressed HR is in agreement with our current data from rats exposed to 100% CO_2 and those of Hirakawa and colleagues.[10] However, the 30% concentration produced only a small, nonsignificant increase in MAP, which is not consistent with the blood pressure observations of Hirakawa and colleagues.[10] This difference could have been due to the O_2 concentration (approximately 14% by calculation) not being as low as it was in the former study (10%)[10] or in our study with 100% CO_2 (approximately 0% O_2). It is interesting that rats appeared not to react as adversely to the 30% CO_2 as they did to the 100% concentration.

It has been proposed that Ar is superior to CO_2 because it is not irritating to animals and therefore does not produce distress. However, we observed that approximately 100% Ar produced significant increases in MAP within the 1st minute of exposure (Figure 4B), suggesting that exposure to high concentrations of Ar may be stressful. In fact, the severe hypoxia or anoxia resulting from breathing 100% Ar is expected to be stressful. If this expectation is true, then exposure to approximately 100% N_2, which also is expected to produce severe hypoxia or anoxia, likely is equally stressful. However, exposure to approximately 100% N_2 did not significantly increase HR or MAP. Therefore, the question of whether Ar exposure is stressful for rats or is operating by some other mechanism requires further experimentation.

The current observations do suggest that Ar can effectively be used to induce unconsciousness of rats, but Ar-exposed rats may show some hyperreflexia and elevated HR for as long as an hour after short-term exposure. The hyperreflexia we detected occurred when animals were handled after they appeared to be unconscious. Therefore, this hyperreflexia may result in continuation of exposure beyond that actually necessary to induce unconsciousness. The associated tachycardia could confound experiments in which HR is being monitored and may indicate suppression of parasympathetic nerve activity, an effect that is consistent with tachycardia without an increase in blood pressure.

The current data from N_2-exposed animals were generally consistent with the observations of Hirakawa and colleagues,[10] who demonstrated that moderate hypoxia and hypocapnia, produced by inhalation of a 90% N_2–10% O_2 gas mixture,

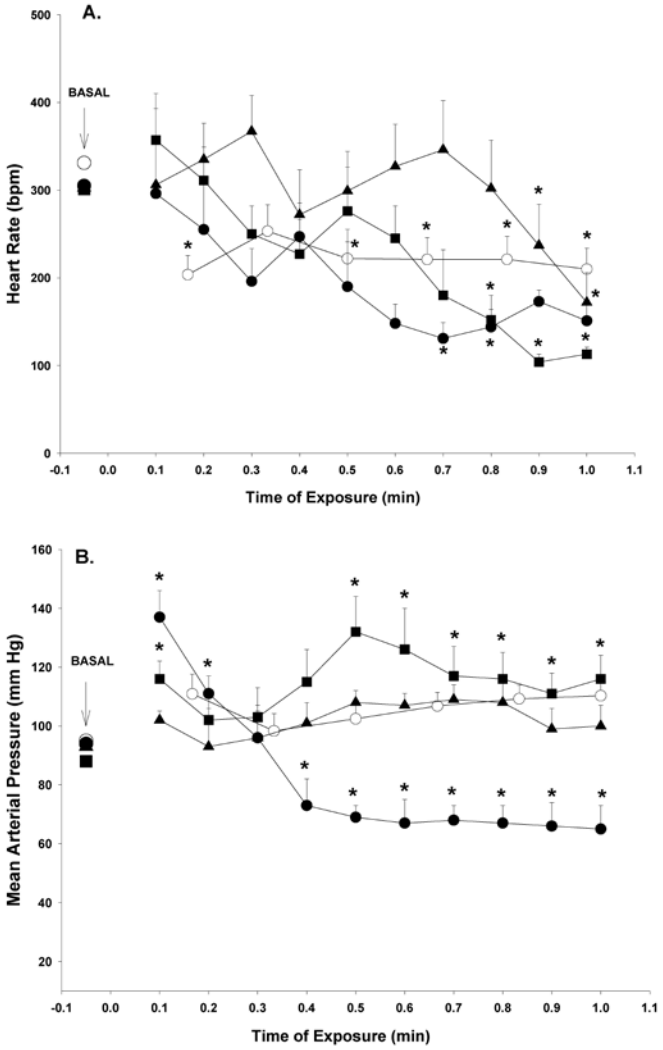

Figure 4. (A) Heart rate and (B) mean arterial blood pressure (mean ± SEM; n = 5 to 9) during the 1st minute of exposure to 100% CO_2 (●), 30% CO_2 (○), Ar (■), or N_2 (▲) in the euthanasia study. *, Significantly different ($P < 0.05$) versus respective control (basal) value.

increased HR of rats but decreased blood pressure. Perhaps the slight differences noted between the 2 studies resulted from differences in the degrees of hypoxia between the current study, in which the O_2 concentration was calculated to be near 0%, and the previous study, in which the O_2 concentration was 10%. It is interesting that exposure to 100% Ar or N_2, two conditions that can be expected to result in the same level of severe hypoxia (or anoxia) and hypocapnia, produced different cardiovascular responses and times to unconsciousness and death (Figures 3 and 4). These observations suggest that these 2 gases do not have the same mechanism of action, a topic that requires further study.

Finally, placing animals in an unfamiliar exposure chamber that contains only room air produces arousal, if not stress (Figures 1 and 2). This effect, although transient if an effective concentration of CO_2 or Ar is established quickly, supports the recommendation that the chamber be precharged. Another method to prevent this effect is to use a system in which the home cage becomes the exposure chamber.

We conclude that CO_2 is effective for inducing unconsciousness and euthanasia, but we have not convincingly answered the question about whether CO_2 induces stress or distress. Ar appears to be effective, but it may be stressful, and it produces effects such as hyperreflexia and tachycardia that may make it less suitable for some studies. We concluded that N_2 is not satisfactory for euthanizing rats or inducing unconsciousness in them.

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