Carbon Dioxide-Induced Anesthesia Results in a Rapid Increase in Plasma Levels of Vasopressin

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Brief anesthesia, such as after exposure to high levels of carbon dioxide, prior to decapitation is considered a more humane alternative for the euthanasia of rodents, compared with use of decapitation alone. Studies of the levels of certain stress hormones in plasma such as corticosterone and ACTH have supported the use of this method of euthanasia in endocrinological and molecular studies. In the current study, rats were briefly exposed to a chamber filled with carbon dioxide until recumbent (20–25 sec), immediately killed via decapitation, and trunk blood collected; findings were compared with rats killed via decapitation with no exposure to carbon dioxide. RIAs were used to measure arginine vasopressin (AVP) and ACTH immunoreactivity (ir) in plasma. Whereas ACTH-ir levels remained steady after brief exposure to carbon dioxide (in accordance with results of other investigators), AVP-ir levels were increased by more than an order of magnitude. These results were confirmed by quantitative capillary-liquid chromatography-mass spectrometry, indicating this observation of rapid increase in plasma AVP-ir levels is not due to nonspecific recognition by the antibody used in the RIA. Likewise, using capillary-liquid chromatography-mass spectrometry, we observed a rapid increase in plasma oxytocin levels after carbon dioxide exposure. These surprising findings have important implications for the design and interpretation of studies involving brief carbon dioxide exposure prior to decapitation as well as those with euthanasia resulting from carbon dioxide-induced asphyxiation. (Endocrinology 150: 2934–2939, 2009)
death after such brief exposure has not been previously addressed. More prolonged exposure to CO₂ as well as hypoxic conditions (low O₂) in hypercapnia/hypoxia studies has been addressed, and large increases in plasma vasopressin were observed (12–14). We report here a dramatic and very rapid increase in the plasma levels of AVP, as measured by RIA as well as capillary-liquid chromatography mass spectrometry, after exposure to CO₂, which serves as a cautionary note for the design and interpretation of studies incorporating CO₂ exposure for the purposes of euthanasia.

Materials and Methods

Animals
Male Fischer and Lewis rats (13–18 wk, 200–250 g) were used in all studies herein and purchased from Charles River Laboratories (Wilmington, MA). Rats were singly housed in stress-minimized rooms, with a 12-h light, 12-h dark cycle, and food and water were provided ad libitum. All animals were housed for at least 1 wk, with daily handling, before studies. Animals were housed and euthanized in a manner approved by The Rockefeller University Institutional Animal Care and Use Committee. Two groups of animals were studied. One was rapidly decapitated immediately upon removal from the home cage and trunk blood collected in ice-cold EDTA-coated tubes. The other group was placed in a chamber that was precharged with CO₂. The characteristics of the chamber were 18 in. (length) × 12 in. (width) × 18 in. (height) plexiglas, with a metal grid approximately 8 in. from the floor. Approximately 1 lb of dry ice pellets were placed on the floor of the chamber and allowed to sublime for at least 5 min with the chamber lid (equipped with small air dry ice pellets were placed on the floor of the chamber and allowed to sublime for at least 5 min with the chamber lid equipped with small air holes for emission of ambient air) closed. The level of CO₂ in the chamber was stabilized for at least 5 min with the chamber lid (equipped with small air holes for emission of ambient air) closed. The level of CO₂ in the chamber after 5 min preequilibration with dry ice was observed to be 100% (±10%), using Gastec calibrated ultrahigh-range carbon dioxide colorimetric detection tubes (Nexteq, Tampa, FL); a similar chamber setup for CO₂-induced recumbency has previously been reported to have a chamber level of 97% CO₂ (8). Animals were monitored until recumbent (defined as animal being prostrate and unresponsive), which for all rats occurred within a time range of 20–25 sec. The animals were then rapidly decapitated and trunk blood collected. Plasma was obtained by centrifuging the tubes at 3100 × g for 15 min at 4°C.

RIA
After collection, plasma was divided into fractions for mass spectrometry (see below), AVP RIA, and ACTH RIA. For ACTH, RIA was performed using untreated plasma, with a kit from Diasorin (Stillwater, MN), in a manner similar to our previous studies (15, 16). AVP RIA has not been used prior by our laboratory, although our laboratory has extensive experience with ACTH RIA (15, 16). The AVP RIA has not been used prior by our laboratory, although it has been used by many other groups. Whereas ACTH levels did not change on this brief exposure of the rats to CO₂, the levels of AVP increased drastically by greater than an order of magnitude (P < 0.005 for both strains of rats).

Mass spectrometry
The fragmentation pattern of AVP analysis was determined using AVP standards prepared in PBS immediately before loading onto the column for mass spectrometric analysis (Fig. 2A). For purposes of quantification of AVP, we used the integrated peak area of a mass chromatogram using ms² tandem mass spectra (precursor ion – vasopressin [M+2H]⁺ = 543.0 m/z, ob-

![Image](https://example.com/image.png)
FIG. 2. Mass spectrometric detection of AVP. A, Fragmentation pattern of AVP: the most prominent fragments resulting from internal cleavage sites correspond to cleavage of the N-terminal cyclic moiety from the C-terminal tripeptide PRGamide, resulting in the y3 and b6 ions. B, AVP standards (0.02, 0.2, 2, and 20 fmol) were loaded in succession onto the capillary-liquid chromatography column and the eluent monitored via tandem mass spectrometry. Ms2, mass spectrometry for the detection of endogenous AVP; if plasma is from rats exposed to CO2. A standard curve of oxytocin in PBS indicated the linearity of integrated peak area of the ms3 product ion 211.2 above 0.5 fmol (Fig. 3B). We observed oxytocin in the plasma of rats exposed to CO2, but not in rats that were not exposed, indicating that the levels of oxytocin present basally in plasma are below the detection limits of the method (Fig. 3C). In comparison with the standard curve of oxytocin (Fig. 3B), the integrated peak areas observed from the plasma of rats exposed to CO2 correspond to at least 10-fold greater than the detection limits, indicating the extent of increase in plasma oxytocin induced by CO2 exposure is likely greater than 10-fold, in concordance with the increase in AVP.

Although we monitored ACTH using ms2 and ms3, we did not detect ACTH in either group of animals, indicating that the plasma levels of ACTH are below the detection limits of the capillary-liquid chromatography-mass spectrometric method (data not shown).

**Discussion**

In our laboratory, investigating stress responsivity in drug abuse, we have been using CO2 before decapitation for a number of years in our studies of rodent models of drug abuse, with measurements of plasma levels of hormones such as corticosterone and ACTH as common end points (15, 16). Recently we observed oxytocin in the plasma of rats exposed to CO2, but not in rats that were not exposed, indicating that the levels of oxytocin present basally in plasma are below the detection limits of the method (Fig. 3C). In comparison with the standard curve of oxytocin (Fig. 3B), the integrated peak areas observed from the plasma of rats exposed to CO2 correspond to at least 10-fold greater than the detection limits, indicating the extent of increase in plasma oxytocin induced by CO2 exposure is likely greater than 10-fold, in concordance with the increase in AVP.

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serving y3 product ion = 328.3 m/z). A standard curve was generated over the range of 20 amol to 20 fmol of AVP in PBS by monitoring the integrated peak intensity from the ms2 mass chromatogram; we observed a linear relationship of the integrated peak area over 3 orders of magnitude (Fig. 2B). Rats exposed to CO2 gave a consistently higher integrated peak intensity corresponding to AVP compared with rats killed without CO2, with representative chromatograms shown in Fig. 2C. Identification of the indicated peak as endogenous AVP was further verified by the ms3 spectrum (data not shown).

In rats that were subject to CO2 anesthesia before decapitation, oxytocin was unambiguously identified in plasma using mass spectrometry, based on the ms2 and ms3 fragmentation patterns (Fig. 3A). The background of the mass chromatogram using ms2 tandem mass spectra (precursor ion – oxytocin [M+2H]+ = 504.5 m/z, observing y3 product ion = 285.2 m/z) was too high for use in the quantification of oxytocin in plasma; hence, we used ms3 tandem mass spectra (precursor ion 1 – oxytocin [M+2H]+ = 504.5 m/z, precursor ion 2 – y3 fragment ion = 285.2 m/z, observing b2 product ion = 211.2 m/z). A standard curve of oxytocin in PBS indicated the linearity of integrated peak area of the ms3 product ion 211.2 above 0.5 fmol (Fig. 3B). We observed oxytocin in the plasma of rats exposed to CO2, but not in rats that were not exposed, indicating that the levels of oxytocin present basally in plasma are below the detection limits of the method (Fig. 3C). In comparison with the standard curve of oxytocin (Fig. 3B), the integrated peak areas observed from the plasma of rats exposed to CO2 correspond to at least 10-fold greater than the detection limits, indicating the extent of increase in plasma oxytocin induced by CO2 exposure is likely greater than 10-fold, in concordance with the increase in AVP.

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fragmentation of vasopressin and oxytocin (Figs. 2A and 3A) are similar to that previously observed by other investigators (21, 22). The standard curve (Fig. 2B) obtained for AVP present in PBS indicates that the integrated signal of the y3 ion (m/z = 328.3) in tandem mass spectra of AVP is a reliable measure of AVP levels. The curve demonstrates linearity of the integrated peak intensity of AVP in the ms2 mass chromatogram greater than 3 orders of magnitude (also found to be linear in another recent mass spectrometric study of AVP) (21). Although relative levels of AVP can be determined, we note that the absolute levels of AVP in plasma cannot be discerned by comparison with the standard curve shown in Fig. 2B; the presence of other peptides in plasma are expected to alter the extent of vasopressin ionization and hence the peak intensities.

The use of mass spectrometry in this instance provided the additional advantage of allowing us to also determine the presence of oxytocin in the blood after CO2 exposure. We did not have oxytocin antibodies or radioiodinated standards immediately available to perform RIA of oxytocin; the mass spectral parameters corresponding to oxytocin were included in the mass spectrometric method essentially as a hunch, given the relationship of oxytocin to vasopressin. Because the baseline levels of oxytocin were not detectable, the extent of increase cannot be accurately determined, although the results of a comparison of standards (Fig. 3B) indicate the oxytocin increase to be at least 10-fold. Although the basal plasma levels of oxytocin and AVP are generally comparable (19), the relative sensitivity of our method is sufficient for detection of basal AVP but not basal oxytocin.
The demonstration of a large increase in plasma oxytocin levels strengthens the notion that CO2 induces a significant change in the hormone levels of plasma and adds a further note of caution in the use of CO2 in the euthanasia of animals. Note that in the case of oxytocin, ms was not sufficient for detection of oxytocin above background, but ms unambiguously shows the presence of this peptide after CO2 exposure. The use of ms for the detection of peptides has similarly been recently used for the detection of endogenous enkephalins (23). Further improvements in sample preparation, including the use of higher levels of plasma, will be investigated with an eye toward the detection of basal levels of oxytocin. No ACTH was observed in any sample using mass spectrometry, indicating the levels of ACTH were below the detection limits of mass spectrometry in both the absence and presence of CO2 exposure, results not inconsistent with the results of the RIA for AVP.

Previous studies have also investigated hypothalamic-pituitary-adrenal (HPA) axis activation through the measurement of ACTH levels after CO2 exposure. After 2 min of exposure to CO2, ACTH levels are increased severalfold (5, 24). Hackbarth et al. (5), in a comparative study, with different times of CO2 exposure, found that ACTH levels had not yet begun to rise after 30 sec of CO2 exposure, in concordance with the results found here (Fig. 1). Several previous studies in our laboratory using similarly brief (<30 sec) CO2-induced anesthesia before decapitation of rodents demonstrated plasma ACTH and corticosterone levels, which are likewise not consistent with pronounced HPA axis activation (15, 16, 25, 26), although it should be noted these studies did not directly compare animals with and without CO2 exposure. It is possible that the delay in CO2-induced ACTH increase is due to a requirement for prior release of an ACTH secretagogue, such as corticotrophin releasing factor; because AVP has been shown to be an ACTH secretagogue (27), it may be that the substantial increase in AVP levels observed here is the primary factor for the rise in ACTH levels observed after 2 min CO2 exposure (5, 24).

Whereas the results of the current study were entirely unexpected, there are reports of investigations that are in concordance with the AVP release observed herein. In addition to the hypoxia/hypercapnia studies in canines (13, 14), which were done using longer exposures, brief CO2 exposure in rats, although generally found to have modest effects on hormonal levels (4), results in significant analgesia, for up to an hour after CO2 exposure (10). The mediator of this analgesia was not determined, but it was shown to be both nonopioidergic, because it was not blocked by naloxone, and dependent on the presence of an intact pituitary gland, because it was blocked by neurohypophysectomy. Because intravenous AVP at high doses in rats induces nonopioidergic analgesia, also for up to an hour (28), the present results suggest that the release of high levels of endogenous vasopressin possibly serves as the mediator of the CO2-induced analgesia.

The investigation of CO2 effects on plasma hormone levels in man (29–34) correspond to some degree with the findings in animals, although the extent of exposure and the time course of plasma sampling are necessarily different. In a study by Kaye et al. (31), exposure to a single breath of 35% CO2 resulted in a significant increase in ACTH in as little as 2 min, similarly to that described in rodents after longer and higher levels of CO2 (5, 24), although the increase is much more modest, as should be expected. These investigators did not find an increase in AVP at the 2-min time point. Certainly there are a number of differences between their study and the current one, including CO2 concentration, exposure duration, sampling time, and species studied, which likely accounts for this difference.

Our finding of a dramatic CO2-induced increase in AVP and oxytocin has important implications for the use of CO2 in methods of euthanasia in studies involving endocrinological measurements. Currently we can but speculate on the potential mechanisms underlying this rapid CO2-induced increase in AVP and CO2. The regulatory mechanism may involve mediation by glutamate (35), catecholamines (36, 37), serotonin (30, 38), or acetylecholine (39), possibly after preliminary activation of carotid chemoreceptors (40). It is possible that hypotension plays a role. Future investigations into the mechanism of CO2-induced increases in AVP may yield insights into other effects of CO2, such as panic-associated behaviors and later increases in HPA activity (29–34, 36).

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