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

Short Title: **CO₂-induced Increase in Plasma Vasopressin**

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Precis: Brief carbon dioxide exposure prior to decapitation leads to dramatic release of vasopressin, and this method of euthanasia is therefore unsuitable for certain endocrinological investigations.

Keywords – Neurohypophyseal hormones; Mass spectrometry; Decapitation; Vasopressin

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Abstract

Brief anesthesia, such as following exposure to high levels of carbon dioxide, prior to decapitation is considered a more humane alternative for the euthanasia of rodents, compared to use of the decapitation alone. Studies of the levels of certain stress hormones in plasma such as corticosterone and adrenocorticotropic hormone (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 s), and immediately sacrificed via decapitation, and trunk blood collected; findings were compared to rats sacrificed via decapitation with no exposure to carbon dioxide. Radioimmunassays were used to measure arginine vasopressin (AVP) and ACTH immunoreactivity (ir) in plasma. While ACTH-ir levels remained steady following 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 radioimmunoassay. Likewise, using capillary-liquid chromatography-mass spectrometry, we observed a rapid increase in plasma oxytocin levels following 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.
Introduction

In the euthanization of animals, various factors must be considered, not least of which is the humane treatment of the animals, but also the safety and comfort of the laboratory workers, and the possible effects of the euthanasia method on any posthumous measurements that may be germane to the study(1). A common method of sacrificing rodents is asphyxiation using carbon dioxide, which has been recommended for some time by the Universities Federation for Animal Welfare and the American Veterinary Medical Association(2,3). In addition to use in asphyxiation-induced euthanasia, exposure to CO₂ for shorter time periods results in anesthesia. Exposure to CO₂ until the animal has become recumbent, followed by rapid decapitation is also a common method for euthanasia, and is more suitable for endocrinological measurements(4). This method has been shown to have minimal effect on the levels of certain hormones related to stress response, with only a slight decrease in the plasma levels of corticosterone and prolactin, in rats(4). Similarly, exposure to CO₂ for thirty seconds did not lead to changes in plasma levels of adrenocorticotropic hormone (ACTH), although longer exposure did lead to elevations(5). The use of anesthetics, including CO₂, prior to decapitation, has been suggested as a more ethical method for euthanasia than decapitation alone(1,4).

Since the initial enumeration of antidiuretic hormone (aka arginine vasopressin, AVP) and its ability to induce antidiuresis and alter arterial pressure, AVP has been intensely studied and characterized, and shown to have a role in various systems(6). A number of early pioneering studies focused on the release of AVP into plasma induced by of pharmacological agents, including anesthetics(7). There have been studies focusing on the effects of CO₂ as an agent in euthanasia, either as an anesthetic prior to decapitation or as the primary inducer of death via asphyxiation, on several behavioral and endocrine parameters(4,5,8-11), but to our knowledge the effect on AVP at the time of death following 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 radioimmunoassay (RIA) as well as capillary-liquid chromatography mass spectrometry, following exposure to CO₂, which serves as a cautionary note for the design and interpretation of studies incorporating CO₂ exposure for the purposes of euthanasia.

Methods
Animals
Male Fischer and Lewis rats (13-18 weeks, 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 hour light/dark cycle, and food and water were provided ad libitum. All animals were housed for at least one week, with daily handling, prior to 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 homecage, 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: 18” (l) x 12” (w) x 18” (h) plexiglass, with a metal grid approximately 8” 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 minutes with the chamber lid (equipped with small air holes for emission of ambient air) closed. The level of CO₂ in the chamber following 5 minutes preequilibration with dry ice was observed to be 100% (± 10%), using Gastec calibrated ultra high range carbon dioxide calorimetric detection tubes (Nextteq, 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 seconds. The animals were then rapidly decapitated, and trunk blood collected. Plasma was obtained by centrifuging the tubes at 3100 x g for 15 minutes, at 4°C.
Radioimmunoassay (RIA)

Following 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 was performed using a kit from Phoenix Pharmaceuticals (Belmont, CA). Plasma (500 μl) was dried down using a vacuum centrifuge, and reconstituted in binding buffer, and the RIA was then performed according to the manufacturer’s instructions.

Capillary-Liquid Chromatography-Mass Spectrometry

Plasma (100 μl) was diluted 1:1 with 2% trifluoroacetic acid and filtered using centricon filters (molecular weight cutoff - 10 kDa; Millipore, Billerica, MA) to remove proteins. Approximately 150 μl was recovered, with 50 μl remaining as the retentate. Capillary liquid chromatography was performed using in-house packed reversed phase capillary columns. The analytical column (75 μm i.d., 5 cm length) was packed using PicoTip Emitter fused silica capillaries (New Objective, Woburn, MA) and 5 μm C18 beads, and the concentrating precolumn (75 μm i.d., 5 cm length) was packed using Integrafrit fused silica capillaries (New Objective) and 15 μm C18 beads. 150 μl of diluted, filtered plasma was loaded onto the precolumn, manually using a pressure bomb. A Pharmacia SMART system delivered a gradient of mobile phase A (1% acetic acid in water) and B (1% acetic acid in 80% acetonitrile/20% water): 0 minutes, 0% B, ramping to 100% B at 17 minutes, holding at 100% B until 23 minutes, returning to 0% B at 28 minutes, followed by a 15 minute equilibration phase. The flowrate of the system was set to 30 μl/minute, with a flow split resulting in column flow of approximately 100 nl/minute, measured using graduated capillaries.

The capillary liquid chromatography system was interfaced with a LTQ ion trap mass spectrometer (Thermo Scientific, Waltham, MA). The mass spectrometric method was set up to record, serially, a ms scan, with a m/z range of 300-2000, followed by data independent ms² and ms³ scans for AVP (ms² precursor ion 1 m/z 543.0; ms³ precursor ion 1 m/z 543.0, precursor ion 2 m/z
328.3), oxytocin (ms² precursor ion 1 \(m/z\) 504.5; ms³ precursor ion 1 \(m/z\) 504.5, precursor ion 2 \(m/z\) 285.2), and ACTH (ms² precursor ion 1 \(m/z\) 764.7; ms³ precursor ion 1 \(m/z\) 764.7, precursor ion 2 \(m/z\) 884.4). In all cases, the collision induced activation level was set to 35.0% according to the manufacturer’s nomenclature, with an activation time of 30.0 ms, and the window for precursor ion 1 was set to a width of \(m/z\) = 2.0, and the window for precursor ion 2 was set to a width of \(m/z\) = 1.0. The mass spectral data was analyzed using the Thermo Scientific software package Xcaliber. For standard runs, AVP was obtained from Phoenix Pharmaceuticals; oxytocin was synthesized by the Rockefeller University Proteomics Resource Center.

**Results**

**Radioimmunoassay**

RIAs were conducted for the measurement of ACTH and AVP in rat plasma, from both Fischer and Lewis rats (Figure 1). 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. While ACTH levels did not change upon 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 (Figure 2A). For purposed of quantification of AVP, we used the integrated peak area of a mass chromatogram using ms² tandem mass spectra (precursor ion – vasopressin \([M+2H]^{2+}=543.0 m/z\), observing 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 three orders of magnitude (Figure 2B). Rats exposed to CO₂ gave a consistently higher integrated peak intensity corresponding to AVP compared to rats sacrificed without CO₂, with representative chromatograms shown in Figure 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 prior to decapitation, oxytocin was unambiguously identified in plasma using mass spectrometry, based on the ms² and ms³ fragmentation patterns (Figure 3A). The background of the mass chromatogram using ms² tandem mass spectra (precursor ion – oxytocin [M+2H]²⁺=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]²⁺=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 (Figure 3B). We observed oxytocin observed 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 (Figure 3C). In comparison to the standard curve of oxytocin (Figure 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 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-LC-mass spectrometric method (data no shown).

**Discussion**

In our laboratory, investigating stress responsivity in drug abuse, we have been using CO₂ prior to 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 endpoints(15,16). Recently, we observed that following exposure to CO₂, the levels of vasopressin in plasma were considerably higher than otherwise unprovoked levels in plasma reported in the literature, which are typically in the range of 0.5 to 5pM(17-19). We therefore compared the effects of CO₂ exposure versus the
absence of CO₂ exposure to investigate this discrepancy. The rapidity and extent of AVP plasma increase are very dramatic, and were unanticipated.

The use of RIA to measure AVP in plasma has a long history, and has been used to show perturbations of AVP levels in plasma due to a variety of stimuli: pharmacological, cardiovascular, osmotic, etc(20). The surprising results presented here, however, raise the possibility of artefactual cross-reactivity. The use of mass spectrometry for the detection of endogenous AVP confirmed that there was indeed a large, dramatic increase in the plasma levels of AVP following brief CO₂ exposure (Figure 2C). The fragmentation of vasopressin and oxytocin (Figures 2A, 3A) are similar to that previously observed by other investigators(21, 22). The standard curve (Figure 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 over three 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 the levels of AVP in plasma cannot be discerned by comparison with the standard curve shown in Figure 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 following 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 (Figure 3B) indicate the oxytocin increase to be at least ten-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 as a further note of caution in the use of CO₂ in the euthanasia of animals. Note that in the case of oxytocin, ms² was not sufficient for detection of oxytocin above background, but ms³ unambiguously shows the presence of this peptide following CO₂ exposure. The use of ms³ 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 towards 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 both in 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 following CO₂ exposure. Following 2 minutes of exposure to CO₂, ACTH levels are increased several-fold (5, 24). Hackbarth et al, in a comparative study, with different times of CO₂ exposure, found that ACTH levels had not yet begun to rise following 30 seconds of CO₂ exposure (5), in concordance with the results found here (Figure 1). Several previous studies in our laboratory using similarly brief (<30 seconds) CO₂-induced anesthesia prior to 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 secretogue, such as corticotrophin releasing factor; as AVP has been shown to be an
ACTH secretogue (27), it may be that the substantial increase in AVP levels observed here is the primary factor for the rise in ACTH levels observed following 2 minute CO₂ exposure(5, 24).

While the results of the current study were entirely unanticipated, there are reports of investigations which are in concordance with the AVP release observed herein. In addition to the hypoxia/hypercapnia studies in canines(13,14), which were done utilizing longer exposures, brief CO₂ exposure in rats, while 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 non-opioidergic, as it was not blocked by naloxone, and dependent on the presence of an intact pituitary gland, as it was blocked by neurohypophysectomy. Since intravenous AVP at high doses in rats induces non-opioidergic analgesia, also for up to an hour (28), the present results suggests 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 timecourse 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 two minutes, similarly to that described in rodents following 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 two minute 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, 39), or acetylcholine (39), possibly following preliminary activation of carotid chemoreceptors (40). It is possible that hypotension plays a role (41). 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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**Figure Legends**

**Figure 1**: Plasma from Lewis and Fischer rats, either decapitated without CO₂ (n=3 for all groups), or exposed for 20-25 seconds prior to decapitation, was used for radioimmunoassays of ACTH and AVP. While AVP showed no change, plasma levels of AVP increased by over ten fold in each strain of rat.

**Figure 2**: Mass spectrometric detection of AVP. **2A** - 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. **2B** - 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 chromatogram - doubly-charged ion of AVP served as the precursor ion, m/z - 543.0 [ms² precursor ion 543.0 m/z; product ion monitoring of 328.2 m/z]. The basepeak was set to correspond to the y₃ product ion (328.3), and the resulting peaks were integrated using Xcaliber software. The integrated peak areas are plotted versus the amount of AVP loaded onto the column; a linear relationship for the amounts tested was found (r² = 0.994). **2C**. Capillary-LC-mass spectrometric detection of endogenous AVP in plasma. Ms² mass chromatograms [ms² precursor ion 543.0 m/z; product ion monitoring of 328.2 m/z] of plasma from representative rats exposed to CO₂ (upper plot) or decapitated without CO₂ exposure (lower plot) of continuous detection, with the base peak set to the y₃ ion, 328.3. Integration of the peaks is indicated by the resulting area under the curve (AUC). The ms² spectra were compared to the spectra from standard AVP (2A) to confirm the peaks shown correspond to authentic endogenous AVP.

**Figure 3**: Capillary-LC-mass spectrometric detection of endogenous oxytocin in plasma. **3A**. Ms² and ms³ spectra of oxytocin in a representative rat exposed to CO₂, with m/z for precursor ions: 504.5 in the ms² spectrum (doubly charged oxytocin), 285.2 in the ms³ spectrum (the y₃ product ion of the oxytocin precursor ion), showing clear correspondence to endogenous oxytocin, with observed ions marked on the
inset peptide backbone fragmentation diagram. 3B. Using oxytocin standard peptide in PBS, we determined the linearity and estimated the detection limits of oxytocin. With 5 fmol, the area under the curve (AUC) of the integrated peak of oxytocin ms³ [precursor ion 1 = 504.5 m/z, doubly charged oxytocin; precursor ion 2 = 285.2 m/z, y3 product ion, product ion monitoring of 211.2 m/z], was 6820. The AUC was linear as the concentration was decreased an order of magnitude, to 0.5 (AUC of 0.5 fmol – 680) (r² = 0.986).

Measurements of AUC below 600 were not reliable, without correspondence to concentration and spectra typically lacking other fragment ions corresponding to the 285.2 product ion (3A). Although the AUC for 0.15 fmol of oxytocin is plotted, it was not included in the regression line. 3C. Ms³ chromatograms [precursor ion 1 = 504.5 m/z, doubly charged oxytocin; precursor ion 2 = 285.2 m/z, y3 product ion, product ion monitoring of 211.2 m/z], for representative rats exposed to CO₂ (upper chromatogram) or not exposed to CO₂ (lower chromatogram). There was no oxytocin detected, using ms³ fragmentation criteria, in the absence of CO₂, whereas we observed oxytocin with AUC of 5770 following CO₂ exposure.