Hyperpolarized [1,4-13C]-Diethylsuccinate: A Potential DNP Substrate for In Vivo Metabolic Imaging

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Introduction: Hyperpolarized ^{13}C MRS provides an unprecedented opportunity for real-time imaging of *in vivo* metabolic pathways.[1] A key component of this emergent field is the development of novel molecular probes that can effectively interrogate these pathways. In particular, the tricarboxylic acid (TCA) cycle has received considerable attention as it plays a central role in cellular metabolism for the production of energy.[2] Furthermore, misregulation of the TCA cycle has been correlated with numerous diseases such as cancer and neurological disorders. Hyperpolarized [^{13}C]-pyruvate has typically been employed as an indirect marker of TCA metabolism. [1-^{13}C]-pyruvate is converted to acetyl-CoA, which enters the cycle, and [1-^{13}C]-bicarbonate via pyruvate dehydrogenase.[3] Although the detection of [1-^{13}C]-bicarbonate indicates flux into acetyl-CoA, this strategy does not provide direct information pertaining to the TCA cycle and intermediates. Alternatively, hyperpolarized [2-^{13}C]-pyruvate, which forms [1-^{13}C]-acetyl CoA and integrates the label into TCA cycle intermediates, has been employed.[4] However with the exception of citrate, only metabolites not on the TCA pathway (i.e. [5-^{13}C]-glutamate and [1-^{13}C]-acetylcarnitine) are regularly observed.

Recently, a novel method was reported for the direct monitoring TCA cycle metabolism via hyperpolarized ^{13}C MRS *in vivo*.[5] This process relied upon the use of a new molecular probe, [1-^{13}C]-diethylsuccinate, which was prepared via parahydrogen-induced polarization (PHIP) from the corresponding [1-^{13}C]-diethylfumarate. [1-^{13}C]-diethylsuccinate, thought to be easily transported across biological membranes, can then be converted *in vivo* to succinate by endogenous esterases. Importantly, this report claimed that various late-stage TCA cycle intermediates (e.g. fumarate and malate) could be successfully observed. Our work explores the synthesis, development and application of [1,4-^{13}C]-diethylsuccinate ([^{13}C]-DES) via DNP polarization. These studies provide a reassignment of the metabolites observed and a reexamination of the role of the TCA cycle in processing [^{13}C]-labeled diethylsuccinate probes.

Methods: The synthesis of [^{13}C]-DES was adapted from a previously published procedure,[5] and the compound can be prepared in spectroscopically pure form with a 66% yield from the commercially available [1,4-^{13}C]-succinic acid (Cambridge Isotopes, Andover, MA). Both *in vitro* and *in vivo* studies were conducted. *In vitro* metabolic studies were performed with the human prostate cancer cell line, PC-3. PC-3 cells were cultured with RPMI media supplemented with FBS/penicillin/streptomycin and grown to 80% confluence prior to experimentation. *In vivo* MR measurements of adult rats (n=3) were performed on a clinical 3-T GE MR scanner with a custom-built ^{13}C transmit/receive surface coil (dia = 28 mm) placed over the heart with rat supine. Healthy male Wistar rats (300-400 g) were anesthetized with 1-3% isoflurane in oxygen (~1.5 L/min), and were injected through their tail veins with 3.0 mL of 80 mM solution of [^{13}C]-DES that had been hyperpolarized using HyperSense DNP (Oxford Instruments). Dobutamine (0.5 mg/kg body weight) was infused i.v. over 10 min before the second [^{13}C]-DES injection to acutely increase cardiac workload.

Results and Discussion: Our initial studies towards the development of the probe were focused in several areas: (1) the toxicity of DES, (2) the hydrolytic stability of the compound and (3) the ability to effectively formulate the agent for DNP polarization. First, no observable toxicity (pulse or respiration) was observed upon i.v. administration of a TRIS-buffered solution containing 80 mM DES. In addition, no detectable ester hydrolysis of DES to yield monethylsuccinate or succinate was observed when DES was exposed to these dissolution conditions for a period of up to 20 min. Lastly, DES was successfully formulated for DNP polarization through the addition of 20 mM α,γ-Bisdiphenylene-β-phenylallyl (BDPA)[6] to 6 M [^{13}C]-DES (neat). The solid-state polarization build-up time constant was 1352 s with a liquid-state polarization level of 5.5%. The T_1 (carbonyls) was 37.9 s in solution.

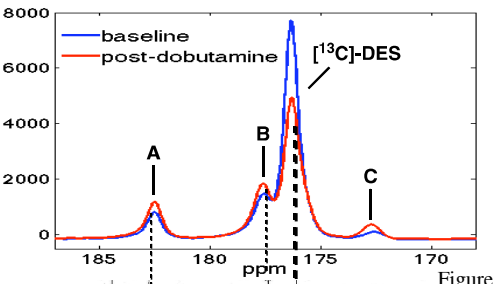

Figure 1. *In vivo* specta of hyperpolarized 80 mM [^{13}C]-DES from rat heart.

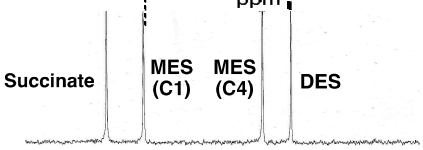

Figure 2. ^{13}C-NMR spectrum of sample containing (2:3:6) succinate:DES:MES. Dashed lines show comparison to Fig. 1.

Fig. 1 displays representative spectra obtained from rat heart after i.v. administration of hyperpolarized [^{13}C]-DES. Dobutamine increases heart rate and myocardial oxygen demand, and is also known to increase flux through PDH and the TCA cycle in rats. The substrate was observed at 176.4 ppm, and three other signals were detected at **A** (182.5 ppm), **B** (177.6 ppm) and **C** (172.7 ppm), respectively. The spectra closely resemble the previously observed product distribution found in the report of PHIP-mediated hyperpolarization of [1-^{13}C]-diethylsuccinate. Despite this similarity, we found several inconsistencies with the previous report's spectral assignments; therefore, we sought to conduct a thorough study of the metabolite distribution. Initially, we analyzed whether the products (monoethylsuccinate (MES) and succinate) correspond to the metabolites observed *in vivo*. In order to conduct this experiment, a sample containing a 2:3:6 ratio of succinate:DES:MES was prepared through treating [1,4-^{13}C]-succinic acid with 1.0 equivalent of trimethylsislyl chloride in ethanol at room temperature (Fig. 2). This sample was then used to reference the MES and succinate carbonyl shifts on a 11.7 T NMR instrument. It was found that peaks **A** and **B** correspond to [1-^{13}C]-MES and [4-^{13}C]-MES, respectively, and do not originate from metabolism via the TCA cycle. In addition, although the conversion of [^{13}C]-DES to [^{13}C]-MES may occur via esterases in the intracellular environment, this process could be catalyzed by enzymes in the blood. When [^{13}C]-DES (100 mM) was added to a freshly drawn rat blood sample, [^{13}C]-MES was clearly formed (Fig. 3). However, no signal corresponding to peak **C** was detected under these conditions.

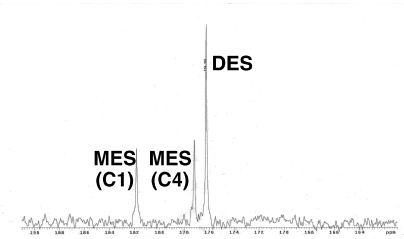

Figure 3. Metabolism of [^{13}C]-DES in blood.

In order to assign peak **C**, we are performing various *in vitro* studies. In order to isolate and characterize the metabolites, PC-3 cells were incubated with media containing [^{13}C]-DES (10 mM). At times ranging from 1 to 60 min, the medium was removed and quenched with methanol. In addition, the adherent PC-3 cells were treated with methanol, scraped from the flask and subjected to further analysis. Examination of the intra- and extracellular fractions by NMR revealed that [^{13}C]-DES was only present in the extracellular samples, which suggests that [^{13}C]-DES was not successfully transported into the cell in detectable quantities. This result potentially suggests DES must be metabolized prior to entry into the intracellular environment. Further *in vitro* studies are ongoing to identify **C**.

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